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Journal of the American Association of Clinical Chemists

VOLUME 1

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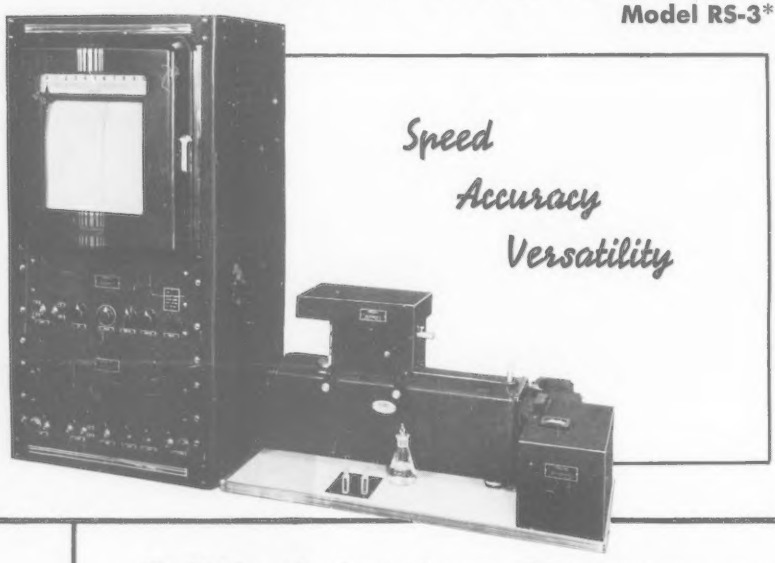

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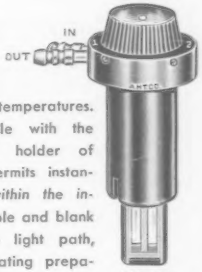

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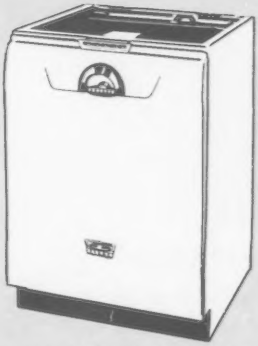

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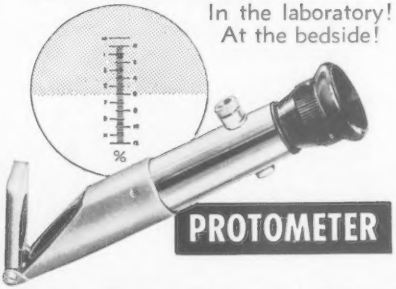

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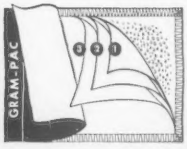

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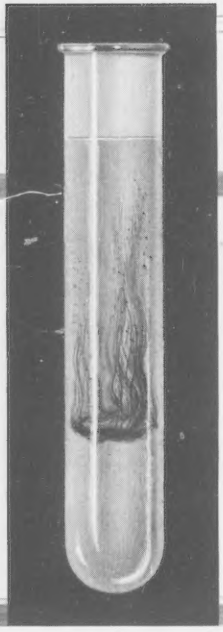

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A NEW JOURNAL

With the appearance of this, the first issue of CLINICAL CHEMISTRY, many hopes and dreams and years of planning come to fruition. Since the founding of the American Association of Clinical Chemists one of its major goals has been the publication of a scientific journal. The constitution defines the objectives of the Association: ". . . to raise the level at which chemistry is practiced in the clinical laboratory; to stimulate the development of new methods for use in the clinical laboratory; . . . to encourage those engaged in this field to pursue advanced studies so as more effectively to render service to the public; and to create and maintain a forum where clinical chemists may exchange ideas and information concerning their scientific, technical and professional problems."

It is the hope of all of us that CLINICAL CHEMISTRY, by fulfilling the last of these objectives, will make easier and more certain the achievement of each of the others.

The practice of modern medicine is based firmly upon the knowledge gained through application of the basic sciences to medical problems. A major portion of this foundation has been contributed by the science of chemistry. The role of chemistry in the rapid evaluation of specific organ function and disease, as an aid in diagnosis and prognosis, and as the foundation for the better understanding of pathogenesis and therapy, can be attributed to the rapid assimilation of new scientific knowledge into the practical analytic methodology and interpretation at the hospital laboratory level.

Reports of this progress have been scattered through the literature of many specialties. It is expected that by providing in these pages a central official medium for the exchange of information, CLINICAL CHEMISTRY will help to bring the entire field into better focus. Thus it will serve not only the active practitioner in the busy clinical laboratory who would like to be aware of new developments as quickly as possible, but also the investigator who will find it easier to keep track of work in other places that may bear on his own studies.

At the outset it is planned to publish material in three general categories. The major share of our space will be devoted to the publication of scientific articles; reports of original investigations of the application of chemistry to medicine, new methodology and laboratory technics, and review articles of current scientific interest. Our Abstract department will offer a guide to current articles of interest to clinical chemists, giving rather complete summaries of new methodology and technics as found in the world's literature. The National Secretary of the Association, as editor of the section, "The Clinical Chemist," will provide notices of events, news of local section and foreign association activities, committee reports, letters from our readers, and all information relating to the operation of the Association and the profession of clinical chemistry.

Other departments can be inaugurated as the need appears. This is only an initial plan. It is hoped that our readers will feel free to offer their ideas and suggestions. The editors are conscious of the fact that, to succeed in their ambitions for this journal, they are dependent on the cooperation of all those actively engaged in the practice of clinical chemistry.

We who have been charged by the National Executive Committee with editing CLINICAL CHEMISTRY are deeply aware of the heavy responsibility that has been placed upon us. In the formative years of a new science a journal devoted to that specialty becomes a showcase and spokesman that is scrutinized with particular care. Our objectives are clear: to help provide answers to the many problems facing those engaged in clinical chemistry; to create and maintain standards of scientific research and writing that will reflect honorably upon our profession; and to provide a continuing forum for discussion of the scientific, technical, and professional problems of all members of our profession. It is our fervent hope that the collaboration of all of us will make CLINICAL CHEMISTRY worthy of the bright future it and its specialty deserve.

HAROLD D. APPLETON
Chairman, Editorial Board

Chemical Abnormalities in Blood Serum Associated with the Carrier State of Viral Hepatitis

John G. Reinhold

APPROXIMATELY ONE in every 100 to 200 persons who receive transfusions of whole blood subsequently become ill of viral hepatitis. When plasma is administered, the incidence of viral hepatitis has been reported to be as high as one in every 5 transfusions, although the usual rate is much lower than this. Since blood for transfusion is collected only from donors who appear to be in good health, it follows that there must be among these donors a fairly large group of apparently healthy persons who harbor the viruses of hepatitis.

There has been no method by which such carriers can be identified until after illness due to viral hepatitis is recognized in the recipients of their blood. Neither has any method for inactivation of hepatitis virus in human whole blood proved to be dependable. The problem of finding ways for reducing or eliminating the incidence of this serious—and sometimes fatal—aftermath of a transfusion is a challenging one, that clinical chemistry may aid in solving.[1] I plan to describe some of the studies made by a group, of which I am a member, working in the Pepper Laboratory.[2] These studies suggest that the risk of viral infection may

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This investigation was sponsored by the Commission on Liver Disease, Armed Forces Epidemiological Board, and supported by the Office of the Surgeon General, Department of the Army.

Presented in part as the Third Ernst Bischoff Lecture in Clinical Chemistry, at the Annual Meeting of the American Association of Clinical Chemists, September 16, 1954. New York City, N. Y.

[1] This topic was chosen as the subject of the Ernst Bischoff lecture because it illustrates the application of chemistry to the investigation of an old clinical problem, the transmission of disease to recipients of blood transfusions. It was chosen also because it provides an example of the work of a team that includes clinical chemists, clinicians, and clinical pathologists.

[2] The group working on this problem has included Drs. D. R. Fitch, D. S. Howell, D.

be lowered if the blood serum of the donor is first examined chemically, by means of the standard chemical tests used for study of liver disease.

The event that led to our work on this problem was the occurrence, exactly four years ago, of homologous serum hepatitis in 3 patients of my colleague Dr. John Neefe. Each had received blood from a single donor. The 3 donors were recalled and their blood was studied chemically to determine whether any abnormality could be found. Results of these tests were clearly abnormal in two of the donors. Encouraged by this finding, other donors whose recipients had developed hepatitis were studied in the same way. These studies led to the segregation of a group of 18 suspect donors. Proof of the carrier state was obtained in 5 out of 6 whose bloods were tested by transmission studies in experiments conducted by Dr. Roderick Murray and his associates of the National Institutes of Health. Descriptions of the circumstances in which hepatitis occurred in the recipients and of the suspected donors have been published recently (1). The transmission experiments are described in Part 2 of the same article (2).

In this paper, chemical studies of proven and suspect carriers extending over several years are reviewed. The possible utility of the tests for the detection of carriers of the virus is assessed and some of the chemical problems encountered in the course of their application are discussed.

METHODS AND DEFINITIONS

Chemical studies have included measurements of serum bilirubin concentrations, cephalin cholesterol flocculation, thymol turbidity and flocculation at *p*H 7.80 and 7.55, zinc sulfate turbidity, cholinesterase activity, and total lipid by means of phenol turbidity. Specimens of urine were tested for bilirubin and urobilinogen. Bromsulfalein retention could not be measured at every visit during the follow-up studies, and the number of tests is about three quarters of that of the other tests. In addition, serum total and esterified cholesterol, albumin and globulin concentrations, and alkaline phosphatase activity were studied in many subjects. Since these seldom differed from limits established in healthy persons, the results are not included in the statistical evaluations.

The term *positive* in the text refers to the result of a test that differs from the normal mean by more than 3 standard deviations. When used

Kassouny, C. B. Mitchell, J. R. Neefe, R. F. Norris, and R. Watanabe, Mrs. L. G. Tourigny, Mrs. Jane Bender, Miss Ann Mifflin, Mrs. Helen Hall, and Mr. John Trumper.

I wish to acknowledge especially the collaboration of Miss Virginia Yonan in chemical aspects of these studies.

in describing the response of a blood donor it indicates that at least 1 test exceeded this limit, or that 2 or more tests gave equivocal results.

Equivocal defines the results differing from the mean by more than 2 standard deviations, but less than 3.

Abnormal results are those that are either equivocal or positive.

Negative indicates that no results have exceeded the 2-standard-deviations limit.

Although the frequency distributions of the measurements made of sera of healthy controls showed skewness when bilirubin, thymol turbidity, and zinc sulfate turbidity were tested, the skewness was not sufficiently marked to alter substantially the usual significance of these limits.

Methods used in these studies were those described in a recent review (3).

CHEMICAL CHANGES ASSOCIATED WITH THE CARRIER STATE

All of the proved carriers repeatedly showed evidence of abnormal liver function. Although occasionally only 1 test was abnormal, this was the exception, and in 21 of 27 studies made at different times 2 or more tests were abnormal. In 12 different studies, 3 or more tests were abnormal, and on 5 occasions, 4 or more tests were so recorded.

The incidence of abnormal liver function tests in the suspect donor group also was high, although not as high as that in the proved carrier group. Seven were examined within 200 days of their blood donation and all showed abnormalities in 1 or more tests. Of the entire group of 13, 7 had at least 2 abnormal test results.

The study of these carriers is continuing and a complete description of the clinical and laboratory studies, extending over 2- to 4-year periods, will be published shortly (4).

Cholinesterase activities were measured with the expectation that low values would be found, in view of the numerous studies that have demonstrated lowered activities in the serum of many patients with parenchymal liver disease. Actually, high values were encountered (Table 1) with much greater frequency than expected. In a group of 176 healthy males, 95 per cent of the serum cholinesterase results were less than 1.17 Michel units (5). Application of the chi square method to the results (Table 2) indicates that the frequency with which high values were observed would have occurred by chance in fewer than 1 of 1000 trials. Two of the higher values were observed among the proved carriers. Only 4 donors of the combined groups showed evidence of a tendency toward low enzyme activities and none were significantly lowered.

Table 1. SERUM CHOLINESTERASE OF PROVED AND SUSPECT CARRIERS

Donor	*Race*	*Days since donation*	*Cholinesterase* (Michel units)	*Status*
11a	White	122	1.44	Suspect
		283	1.17	
9a	White	287	1.35	Suspect
		561	1.06	
2	Negro	114	1.28	Proved
		385	1.12	
10b	White	226	1.22	Suspect
3	Negro	68	1.19	Proved
		97	1.27	
		357	1.07	
6	White	101	1.07	Suspect
		128	1.13	
1	White	203	1.03	Suspect
9b	White	294	0.84	Suspect
5	Negro	221	0.80	Proved
7	Negro	85	0.80	Proved
		136	0.69	
		241	0.87	
13a	White	130	0.78	Suspect
		152	0.82	
8	White	43	0.67	Proved
		62	0.73	
4	Negro	114	0.64	Suspect

Search of the literature has disclosed a few instances in which high serum cholinesterase activities were reported. Kark (6) found high values in patients recovering from cirrhosis of the liver. Experimentally, a diet poor in lipotropic factors has been found by Hawkins and Nishikawara (7) to cause a rise in plasma and liver cholinesterase of male rats. However, female rats responded in an opposite manner.

Yet another finding in this group of carrier-donors is the frequent occurrence of elevated concentrations of lipid in serum. Lipid concentrations were measured by the phenol turbidity test of Kunkel, Ahrens, and Eisenmenger (8).

Table 2. Serum Cholinesterase of Proved and Suspect Carriers of Viral Hepatitis

	Michel units		Chi square[a]
	<1.17	>1.17	
Healthy males	171	5	24.3
Carriers	8	5	

[a] Corrected for continuity.

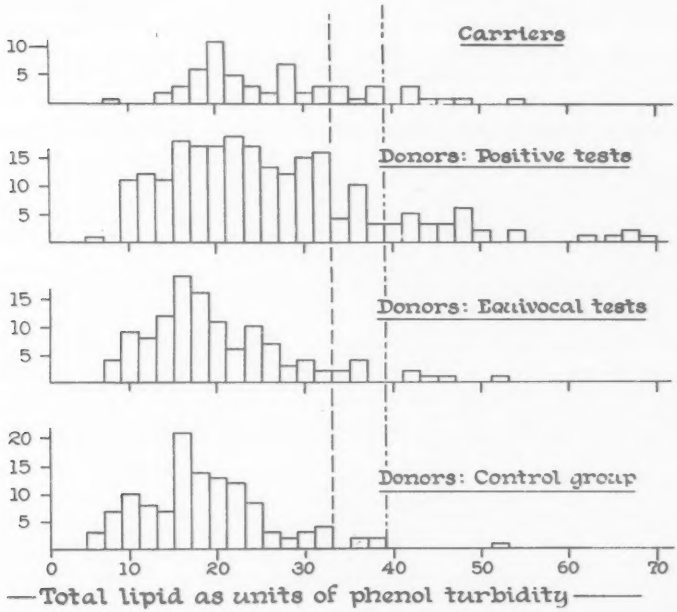

Fig. 1. Serum total lipid of donor-carriers and of blood donors with abnormal hepatic tests compared with those whose tests were normal. The ordinate shows the number of persons whose serum lipids were within the limits shown on the abscissa. The broken lines indicate the mean plus 2 and 3 standard deviations respectively.

Figure 1 shows the frequencies (on the vertical axis) with which serum lipid fell within certain limits in the carrier-donors and in a group of blood donors classified as positive on the basis of their hepatic tests. The similarity of the distributions of these two groups, shown in the

upper half of the figure, is evident. It is clear that these differ from the other two shown, one of which includes the results found for a group of donors showing slight abnormalities (the equivocal group). Not only are the modes of the carrier-donor and positive groups shifted toward higher values, but one third of the analyses in the carrier-donor group gave results that were abnormally elevated. This is a frequency that is about six times the expected. The frequency of high values in the positive group was five times the expected, and that of the equivocal group about twice the expected rate. The tendency toward hyperlipemia is important in connection with the use of the thymol test for screening blood donors, as will be shown later in this paper. Supporting evidence of the existence of hyperlipemia has been provided by use of more specific methods for determination of lipids, and a more complete report will follow.

EVALUATION OF THE BEHAVIOR OF CERTAIN HEPATIC TESTS

The prevalence of chemical abnormalities in the blood of those donors believed to be harboring the viruses of hepatitis suggests that application of appropriate chemical studies frequently would have indicated that the blood was unsuitable for administration.

Table 3 summarizes the results of the chemical studies of the proved and suspect carriers over a period of approximately 2 years. Bromsulfalein retention and thymol turbidity (done at *p*H 7.55) head the list with 76 and 78 per cent of positive tests in the group of proved carriers. The zinc turbidity also stands high in the list but approaches the frequency of abnormal thymol tests only when the equivocal group

Table 3. Frequency of Abnormal Hepatic Tests Given by 5 Proved and 13 Suspect Carriers[a]

Test	*Proved carriers*			*Suspect carriers*		
	No. of tests	*Equivocal (%)*	*Positive (%)*	*No. of tests*	*Equivocal (%)*	*Positive (%)*
Bromsulfalein	17	0	76	17	12	30
Thymol turbidity	27	0	78	36	11	25
Thymol floc	27	4	70	36	3	6
Zinc turbidity	27	15	60	36	11	8
Cephalin cholesterol floc	27	4	30	36	0	3
Bilirubin, serum	18	28	6	36	8	3
Bilirubin, urine	13	0	0	30	0	0
Urobilinogen, urine	13	23	8	28	7	7

[a] All tests are included.

Table 4. Frequency of Abnormal Hepatic Tests during First 200 Days

Test	Proved carriers			Suspect carriers		
	No. of tests	Equivocal (%)	Positive (%)	No. of tests	Equivocal (%)	Positive (%)
Bromsulfalein	6	0	100	8	12	38
Thymol turbidity	13	0	84	16	19	31
Thymol floc	13	8	70	16	0	0
Zinc turbidity	13	16	61	16	19	13
Cephalin cholesterol floc	13	16	8	16	0	6
Bilirubin, serum	13	16	8	14	7	7
Bilirubin, urine	8	0	0	15	0	0
Urobilinogen, urine	8	12	0	15	7	13

is included. The other tests were less frequently positive. No abnormality was detected by the test for bilirubin in urine. The ranking of these tests in the suspect carrier group is similar although the proportion of abnormalities is much lower.

Perhaps a better standard for evaluating the possibilities offered for the detection of undesirable donors is shown in Table 4 which lists the results of studies made during the first 200 days following the donation of blood associated with the development of hepatitis in the recipient. The ranking of the tests remains about the same. The bromsulfalein retention was abnormal in all of the proved carriers tested during this period, and in 4 of the 8 of the suspect group.

The testing of all prospective blood donors would require that the test or tests used be selective, technically simple, rapid, and inexpensive. The first consideration clearly must be the ability of the tests selected to detect carriers of viral hepatitis. Although no single test or group of tests is capable of doing this unfailingly, some have a much better ability to do so than others. Evidence concerning the contribution of each of the various tests is shown in Table 5.

If we assume that each separate application of a group of tests represents an attempt to detect a carrier presenting himself at a Donation Station, it will be seen that abnormalities were detected in each of 27 such trials in the 5 proved carriers. If the bromsulfalein test is omitted, abnormalities were found in 23 of the 27. Omission of the test for serum bilirubin and zinc turbidity would have permitted 1 more carrier to escape in each instance, but omission of tests for urobilinogen and the cephalin cholesterol flocculation test would have made no difference. A total of 21 abnormal results were found by either the thymol test or the zinc turbidity.

Table 5. Effectiveness of Hepatic Tests in 5 Proved and 13 Suspect Carriers

	Number of trials remaining abnormal[a]		*Number of trials remaining positive*	
	Proved	*Suspect*	*Proved*	*Suspect*
All tests included	27	24	26	17
Bromsulfalein omitted	23	21	21	15
CCF omitted	23	20	21	14
Urine urobilinogen omitted	23	18	21	13
Serum bilirubin omitted	22	15	21	12
Zinc turbidity omitted	21	15	20	11
Thymol turbidity alone	21	15	20	11
Total number of trials	27	36	27	36

[a] "Abnormal" includes both equivocal and positive.

The experience with the suspect group is similar (Table 5). Of the 36 trials, 1 or more tests were abnormal in 24, with 21 remaining abnormal after the bromsulfalein test was omitted. Cephalin cholesterol flocculation and urine urobilinogen enabled detection in 1 and 2 trials respectively, and serum bilirubin in 3. Fifteen were detected by the zinc or thymol turbidity tests alone. However, the zinc turbidity test was equivocally abnormal in a larger proportion than was the thymol test, therefore it was concluded that the thymol test was the method of choice if a single test was to be used, with others to be included if possible.

APPLICATION OF HEPATIC TESTS TO LARGE-SCALE EXAMINATION OF BLOOD DONORS

We have applied these observations to the screening of blood donors (9). A study of 3655 donors by means of the tests listed in Tables 2 to 4 showed an unexpectedly high proportion of abnormal results, approximately 12 per cent being classified as having positive tests and 17 per cent as equivocal. The thymol test accounted for most of the abnormality encountered, and it was soon observed that many of the abnormal readings of the thymol test were occurring in serum specimens that were lactescent. It is well known that the thymol-barbital reagent reacts with serum lipid, but we believed that by using standard values established in a large and well-matched group of nonfasting controls, difficulty from this source would be eliminated.

That the association of lactescence in the serum with abnormality in thymol turbidity reading is not consistently one of cause and effect

Table 6. Relationship of Lactescence to Thymol Turbidity

Group	Appearance of serum	No. of sera	Thymol turbidity		
			Negative	Equivocal	Positive
Unselected donors	Clear	543	530	3	10
	Slight turbidity	233	219	7	7
	Lactescent	269	172	37	60
Total sera		1045	921	47	77
Equivocal group	Clear	9	9	0	0
	Slight turbidity	9	7	2	0
	Lactescent	10	6	4	0
Total sera		28	22	6	0
Positive group	Clear	15	12	0	3
	Slight turbidity	8	4	1	3
	Lactescent	36	18	4	14
Total sera		59	34	5	20

may be seen in Table 6. This shows the frequency with which abnormal thymol turbidity readings were encountered in unselected blood donors as compared with donors previously tested and classified as positive. About 25 per cent of the 1045 sera from the unselected donors were lactescent, but of these only one third had abnormal thymol turbidities. Abnormal thymol turbidities occurred in lactescent sera about ten times as often as in clear or slightly turbid sera.

Among the donors classified as positive, lactescence was recorded in 61 per cent. Abnormal thymol turbidity was observed in half of these. Although the proportion of lactescent specimens found to be positive by the thymol test in this group was greater than that of the unselected group, the difference was not statistically significant (chi square 2.63, $p = 0.10$). On the other hand, the frequency of positive tests in the clear and slightly turbid specimens was significantly greater in the positive group. However, if the high thymol turbidity readings of all lactescent sera were to be rejected as artifacts, a significant number of donors with abnormal liver function tests would not be excluded, especially if the thymol test was used as the single screening test. Furthermore, several of the proved and suspect carriers then would have escaped detection on each of several trials.

Lactescence conceivably may occur more frequently in persons suffering from viral hepatitis or in those who have residual effects from hepa-

titis. Elevated concentrations of serum lipid and shifts in distribution of lipoprotein occur following experimentally produced liver damage, as Pierce and Gofman (10) have demonstrated. These authors and Pierce, Kimmel, and Burns (11) have found such changes in viral hepatitis also. Woldow and his associates (12) recently observed abnormalities in serum lipid, characterized by elevated values after a fat meal, several years after recovery from an attack of hepatitis. Mar et al. (13) at times observed high neutral fat concentrations in hepatiti , and De la Huerga and his associates (14) reported high serum tota. lipids. A tendency toward high concentrations of serum lipids in p tients with hepatitis may escape notice because of the low fat intake .sually prescribed for patients known to have liver disease.

Although lactescence and hyperlipemia may reflect disturbances of liver function, their occurrence in the sera of blood donors usually is the result of ingestion of fatty food a few hours before their donation. Specific instructions that fatty foods be avoided on the day of blood donation have led to a substantial lowering in number of lactescent sera

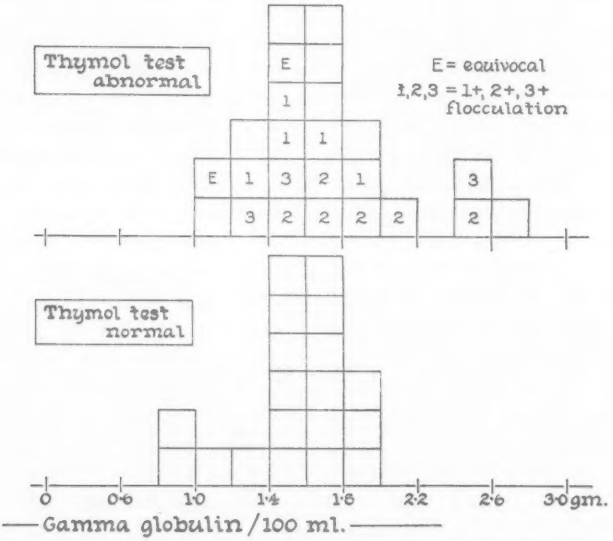

Fig. 2. A comparison of serum gamma globulin concentrations of blood donors with abnormal hepatic tests with the gamma globulin concentrations of those whose tests were normal. Each square represents 1 donor.

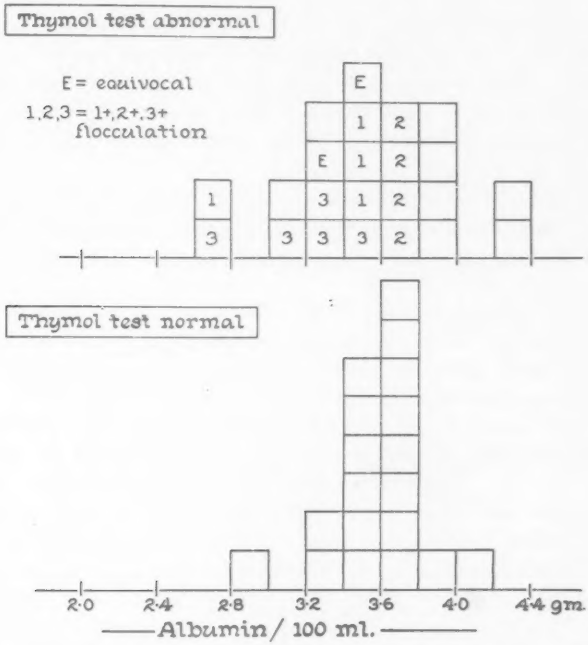

Fig. 3. A comparison of serum albumin concentrations of the same donors as in Fig. 2.

encountered in the Blood Bank.[3] Currently, thymol turbidities falling within the "positive" classification number approximately 7 per cent. This remains much higher than the expected incidence of abnormality based on the rate of clinically recognized viral hepatitis transmitted by blood transfusion, which is 0.5 to 1 per cent. The discrepancy is explained in part by the occurrence of other undetected diseases in blood donors (15).

Because of the question raised concerning the significance of the abnormal thymol tests given by blood donors, we recently selected at random 24 donors with positive thymol turbidity tests and examined their sera by means of zone electrophoresis. Figure 2 shows that 4 of the 24 had significantly elevated gamma globulin concentrations judging by the distribution of concentrations found in a similar group of donors

[3] Another method of partially circumventing the dietary lactescence factor is the use of clarified serum obtained by centrifugation at reasonably high speeds.

whose hepatic tests were normal. Gamma globulin concentrations of others in the group closely approached the upper limit defined as normal.

Figure 3 shows a similar comparison of albumin concentrations of the 2 groups. Four of those with abnormal thymol tests had significantly lowered albumin concentrations, 2 being donors who did not show abnormal gamma globulin. One of the control group also showed an abnormally low albumin concentration.

Beta globulin concentrations are shown in Fig. 4. Although there are no individual deviations in this fraction, the entire group with the abnormal thymol test results shows a shift toward lower values. Thus, the

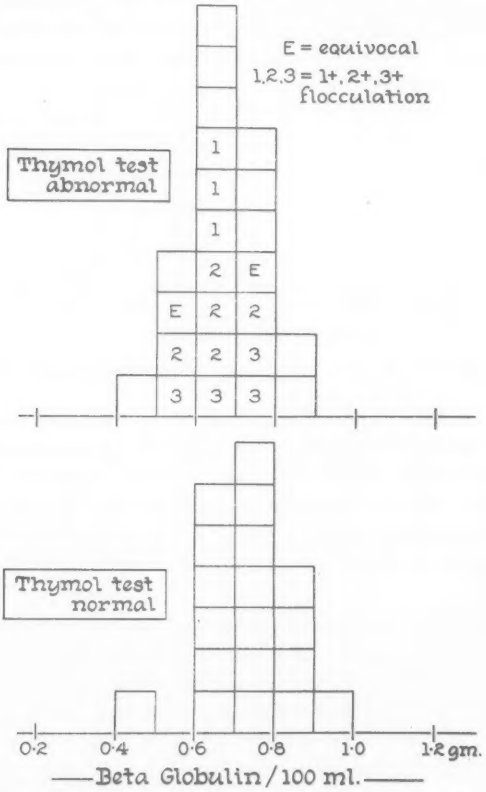

Fig. 4. A comparison of serum beta globulin concentrations of the same donors as in Figs. 2 and 3.

results of the zone electrophoresis study demonstrate significant abnormalities, similar to those frequently observed in liver disease, in 6 of the 24 members of the group with abnormal thymol test responses, while the group as a whole seems to differ in its beta globulin composition. Failure to demonstrate abnormalities in the serum protein distribution of the remainder does not exclude liver damage, for abnormal thymol turbidity and flocculation readings may occur in liver disease despite apparently normal protein analysis.

Results Of Blood Donor Screening

Some evidence of the value of blood donor screening has been gained during the past 4 years in applying hepatic tests to donors reporting to the Donation Station of the University Hospital. During the first 2 years, blood was issued from the Blood Bank without knowledge of the test results. Five patients who had received this tested blood were discovered to have developed viral hepatitis (9). In each instance, the hepatic tests of at least 1 donor were abnormal.

During the past 2 years, bloods whose donors had thymol turbidity readings exceeding 6.6 units have not been used for transfusion. Analysis of the results, insofar as incidence of viral hepatitis is concerned, of the transfusions of the bloods giving acceptable readings has not yet been completed, in part because of the difficulties of learning the fate of recipients of specific bloods. Besides, definitive evaluation of a screening program must be based on a very large number of thoroughly studied donors and recipients.

The success with which hepatic tests may be used for identifying an occasional carrier among large numbers of healthy donors requires that careful attention be given to technic and to the use of appropriate standards. It became evident as we applied the thymol test to screening of blood donors that both the accuracy and the precision of the existing technics were inadequate. Investigation of the cause demonstrated that the temperature at which the reaction was carried out was critical, and consequently that temperature control was essential (16). An improved method of preparing the reagent also was developed of necessity (3). It is essential also that correct limits defining the normal be applied because of the several different units of measurements, and because such limits differ for postprandial as compared with fasting specimens. We consider thymol turbidities exceeding 5.5 to be equivocal and those exceeding 6.6 (Shank-Hoagland) units to be positive when postprandial sera are examined.

CONCLUSIONS

We recognize that the screening of donors by means of the thymol test or even by a group of hepatic tests will fail to detect some carriers of viral hepatitis. It leads also to the rejection of a substantial number of blood donations and so adds to the costs of transfusion. Whether the improvement in safety of transfusion is worth this added cost remains to be established. However, viral hepatitis is a serious disease and even a moderate lowering of its incidence would justify a considerable outlay. Several other factors should be taken into account in an evaluation of donor screening. It has led to the detection of a significant number of other diseases including diabetes, peptic ulcer, and miscellaneous conditions in a group of 120 donors whose blood was rejected for transfusion because of positive tests (15). Furthermore, the blood withheld because of abnormalities in hepatic tests can be salvaged in one of several ways. The plasma may be separated and stored at room temperature for 6 months, as recommended by Allen *et al.* (17). Although Murray *et al.* (18) found 6 months of storage insufficient for completely inactivating the viruses of hepatitis present in considerable concentration, there is good reason to believe that a few additional months of such storage would do so. As another alternative, the plasma could be used for albumin preparation since albumin withstands the application of heat for a sufficient time to permit destruction of these viruses.

Additional information is needed about carriers of viral hepatitis so that hepatic tests may be applied to the screening of blood donors with greater confidence. One should be alert for the occurrence of viral hepatitis following blood transfusion especially in recipients of blood from single donors because in these the evidence of transmission is strong. Such donors should be studied thoroughly and warned not to give blood henceforth to prevent further spread of infection.

Our findings demonstrate that many carriers of viral hepatitis can be recognized by means of chemical studies. Concentrated efforts of chemists, physicians, and scientists from other fields undoubtedly will diminish still further the hazard of viral hepatitis as a complication of blood transfusions.

SUMMARY

The results of studies made for the purpose of identifying carriers of viral hepatitis among blood donors have been reviewed. Some carriers of the virus appear to suffer from a subclinical liver disease recognizable

by abnormalities in hepatic tests. The carriers of the virus also tend to have elevated serum cholinesterase activities and elevated serum lipid concentrations.

The use of hepatic tests for screening large numbers of blood donors has been discussed in the light of observations made of the behavior of hepatic tests in proved and suspect carriers. Evidence is presented that among the donors with abnormal hepatic tests there are some that show changes in serum protein as measured by zone electrophoresis, characteristic of liver disease.

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Electrophoresis and Monomolecular Layer Studies with Serum Lipoproteins

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THE LIPIDS OF BLOOD SERUM may be classified as follows: triglycerides, phosphatides (predominantly with a P/N ratio of 1:1), and cholesterol, in the free and in the esterified form. There are in addition small amounts of free fatty acids, acetal phosphatides, phosphatidic acids, carotenoids, and lipid hormones and vitamins, which we shall, however, neglect in considering the over-all quantitative viewpoint. The major portion of the serum lipids is bound to certain protein fractions, forming the so-called lipoproteins. Whether the complexes between lipids and proteins are held together by absorptive forces or by main or coordinative valencies, particularly those of the phosphoric acid and the nitrogenous components of the phosphatides, has not been decided. Both mechanisms may coexist (1).

Most of the bound lipid occurs in the α- and β-globulin fractions, much smaller amounts being associated with albumin. A breakdown of these lipoproteins produces visible heterogenicity, the liquid assuming a milky or creamlike appearance, as the lipids, hitherto in colloidal solution, form an emulsion. This change may be brought about in normal sera by incubation with the so-called α-toxin of *Clostridium welchii*, an enzymatic factor which hydrolyzes lecithin into the diglyceride and the phosphoryl-

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choline moieties. This disruption of the phosphatide, the chief mediator in the complex formation between protein and fat, is sufficient to set free the electroneutral lipids, the triglycerides and sterols, which give rise to lactescence even at the relatively low concentrations, below 1 per cent, found in normal serum. Even without addition of an enzyme preparation, lipoprotein may break up spontaneously by an endogenous enzymatic reaction in the course of months, a problem encountered in the manufacture of antitoxins and antisera.

In certain pathologic and experimental conditions, the amounts of lipids, particularly of the non-ionizable types—the glycerides and cholesterol—exceed the capacity of the globulins to carry them, and in these cases the fresh serum or plasma in the fasting state appears opaque or milky. Lactescent sera, including those observed after a fatty meal, contain microscopically visible lipid aggregates, the chylomicrons. These will be considered as part of the neutral fat fraction in the following discussion.

EXPERIMENTAL PROCEDURE

Paper Electrophoresis

Chemical analysis of the lipid partition was carried out by the customary methods. The lipid phosphorus, determined by Sperry's modification (2) of the method of Fiske and Subbarow, was multiplied by the factor 25 to give the amount of the total phosphatides. Total and esterified cholesterol were determined according to Schoenheimer and Sperry (3). For the calculation of the neutral fat we subtracted from the total lipids the phosphatides and the total cholesterol plus 68.5 per cent of the amount of esterified cholesterol, to make allowance for the combined fatty acids. This correction for the fatty acids was not taken into account in our previous publications (4, 5, 6, 7). The average fatty acid molecular weight, 282, bears the ratio 68.5:100 to 382, the M.W. of cholesterol. This procedure gives an adequate picture of the chemical nature and quantity of the most important serum lipids.

The lipoproteins were studied by zone or paper electrophoresis (8, 9, 10, 11). All sera were obtained in the fasting state and examined either fresh or after deep-freeze storage. We have found in separate experiments that storage in the frozen state for as long as 6 months does not affect the results. Serum was examined in man, heparinized plasma in the experimental animals.

We have selected an apparatus with horizontally suspended paper strips. We used Whatman filter paper 3MM, 45 cm. long and 2.5 cm.

wide, moistened with 0.06M veronal buffer of *p*H 8.6 and ionic strength 0.05. We applied 0.005 ml. serum or plasma to a pencil-ruled mark across the center of the paper for the protein determinations. Six to twelve times this amount, 0.03–0.06 ml. serum, was used for the lipoprotein analysis. This concentration range does not affect the rate of migration. A current of 250 v. and 10 to 15 ma. was applied for 7 hours, after which the paper strips were dried in an oven at 70° for 40 minutes.

The protein patterns are evaluated by staining the proteins with Amidoblack 10 B and washing away the excess dye with 30% acetone.

Bromophenol Blue, in our experience, gives satisfactory visual patterns, but unreliable densitometric readings. With this dye the albumin is less chromogenic, giving rise to erroneously low optical-density readings (12).

The parallel strips used for lipid analysis were stained with a saturated solution of Oil Red 0 in 60% ethanol and the excess dye removed by rinsing with tap water (13).

A satisfactory background color could not be obtained with Sudan Black and the great variations in uptake of the stain prevented satisfactorily reproducible results. The uptake of Sudan IV (14), by serum lipids was found to be only about half that obtained with Oil Red 0.

With Oil Red 0 the densitometric readings checked closely with results obtained by elution and colorimetry. Densitometry of the papers which were not made transparent prior to densitometry was carried out in a Photovolt Model 525 instrument. Optical-density readings were recorded at millimeter intervals. The resulting curves were analysed by planimetry after components were separated by perpendiculars dropped to the baseline from the troughs of the curves. Proteins and lipid patterns of the same serum were plotted on one graph for comparison of the resultant curves. The protein pattern provides a guide for the allocation of the lipoprotein components.

Observations in Normal Mammals

The combined method of lipoprotein determination by paper electrophoresis and of serum lipid levels by chemical analysis was first used in a comparative study of normal man and laboratory animals. The purpose of the study was to characterize each species under normal conditions prior to the study of pathological or experimental states. Table 1 includes the average values from observations in 19 normal adults below the age of 50 without clinical evidence of disease, 5 rhesus monkeys, 8 male dogs,

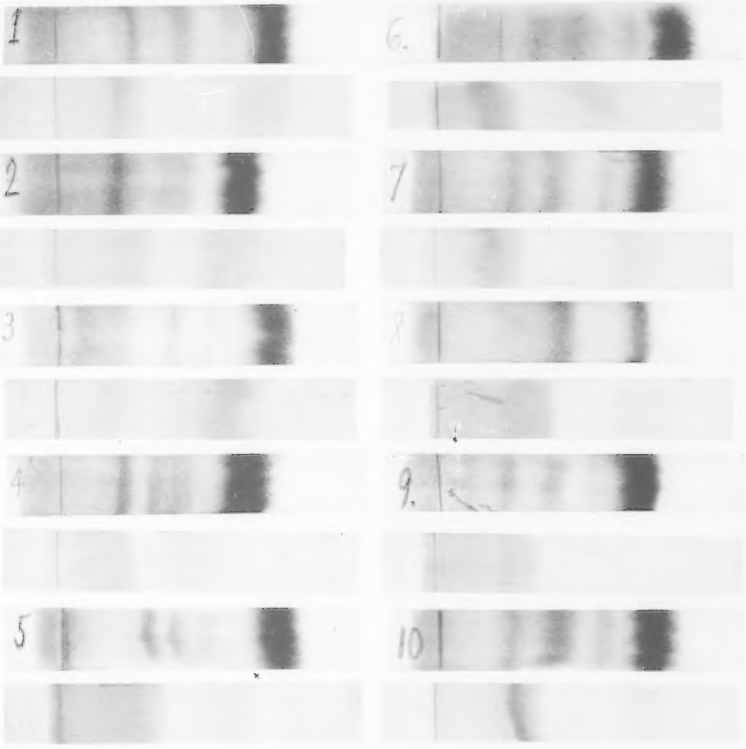

Plate I. The illustrations of the stained paper strips on the plate correspond to the electropherograms, which were derived from them as indicated in the text. The blue strips show the protein partition, stained with Amidoblack 10 and the pink strip beneath each protein strip gives the lipids stained with Oil Red O. Note that fractions on the paper strips migrate from left to right. The site of application of the serum is indicated by a perpendicular line. The albumin fraction is at the right. These strips therefore are mirror images, as it were, of the corresponding electropherograms in Figures 1–10.

Table 1. Lipid Partition and Lipoprotein Patterns

Species	*Total lipids* (mg./100 ml. serum)	*Neutral fat* (mg./100 ml. serum)	*Phosphatides* (mg./100 ml. serum)	*Cholesterol* (mg./100 ml. serum)		*Percentage of sudanophilic lipids*		
				Total	*Est.*	$\alpha_1 + \alpha_2$	β	"0"
Man	860	275	260	215	160	35	52	13
Monkey	780	290	280	140	105	41	41	18
Dog	785	190	315	190	135	70	22	8
Rabbit	440	230	120	60	45	32	50	18

and 15 male rabbits. This and the following tables give on the right-hand side the protein-bound lipid in percentage of the total lipid stainable after paper electrophoresis. Examples are given in Figs. 1–4 and in Plate I.

It must be emphasized that the evaluation of lipids on electropherograms gives approximate values only, for a number of reasons: (a) considerable adsorption of low-density lipoproteins (chylomicrons) and of β-lipoprotein to the paper interferes with the resolution of the lipid spectrum (15, 16). Electrophoresis on starch circumvents this difficulty (15, 17); (b) the direct densitometry of the stained paper, whether by transmission or by reflection, does not follow Beer's law over the whole range; (c) the chromogenicity of the dyes for the various lipids and sterols is in general not proportional to their weight; (d) lipids in mixtures may influence each other's chromogenicity. Figures obtained by this method may be used for guidance and comparison, but the validity of the absolute values remains to be established.

Amongst the lipids only the phosphatides contain ionizable groups and will thus travel with the electric current, both in the free or combined state. On the other hand, the triglycerides and cholesterol, in the free as well as in the esterified form, will remain stationary in the electric field, unless engulfed by charged molecules. The last column, inscribed *0*, gives the percentage of lipids which do not migrate, but stay at the point of origin because of their electroneutrality or their adsorption on the paper. This fraction, composed of triglycerides including chylomicrons and trailing β-lipoprotein, will greatly overlap, but not in general be identical in quantity, with the neutral fat fraction, as estimated by chemical analysis.

Man, compared to the other species studied, is characterized by high levels of cholesterol, with a phosphatide:cholesterol ratio of 1.2:1. Man's serum lipoprotein profile shows comparatively low α- and high β-globulin-bound lipids.

The dog's plasma lipids are characterized by an average cholesterol level slightly lower than in man, while the phosphatides are higher, resulting in a phosphatide:cholesterol ratio of 1.7:1. The total lipids and neutral fat in dogs are very close to the human level, but the lipoprotein profile is exactly the opposite to that of man; the α-fraction represents 70 per cent and the β-fraction 22 per cent of the total.

The plasma lipid levels of the monkey are slightly lower, while those of the rabbit are considerably lower than the values obtained in man and dog. The phosphatide:cholesterol ratio in these vegetarian species averages 2:1. The α- and β-globulin–bound lipids in monkey are both 41 per cent, while the figure for the rabbit approach those of man.

The species examined thus show distinctive patterns which can perhaps be correlated with the observed differences in their propensity to atherosclerosis. These pathologic changes are known to occur in man and rabbit, when their cholesterol levels are raised. The dog is strikingly resistant in this respect and only complete suppression of thyroid function, resulting in marked elevation of plasma cholesterol, will break this resistance. In the monkey atherosclerosis can be produced by cholesterol feeding and deficiency of sulfur-containing aminoacids. These observations suggest a correlation between predisposition to spontaneous or induced atherosclerosis and high levels of β- and low levels of α-lipoproteins.

A comparison of the protein patterns in the various species reveals differences in mobility of albumin: the canine albumin fraction moves most

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Fig. 1. Electropherogram of normal man. The solid lines represent the protein, the dotted lines the lipid profiles, the arrows in the right lower corner indicate the point where the serum was applied. The protein peak farthest to the left represents albumin, the fastest moving component, followed to the right by α-, β-, and γ-globulin, and fibrinogen in Figs. 3, 4, 5. The lipid peak farthest to the left represents the α-fraction, followed by β- and "0"-fractions. The distance is given by notches on the abscissa for each 3 cm., the optical density by notches on the ordinate for 4 units each.

Fig. 2. Electropherogram of normal monkey. For explanation see caption to Fig. 1.

Fig. 3. Electropherogram of normal dog. For explanation see caption to Fig. 1.

Fig. 4. Electropherogram of normal rabbit (see table 1) for explanation see caption to Fig. 1.

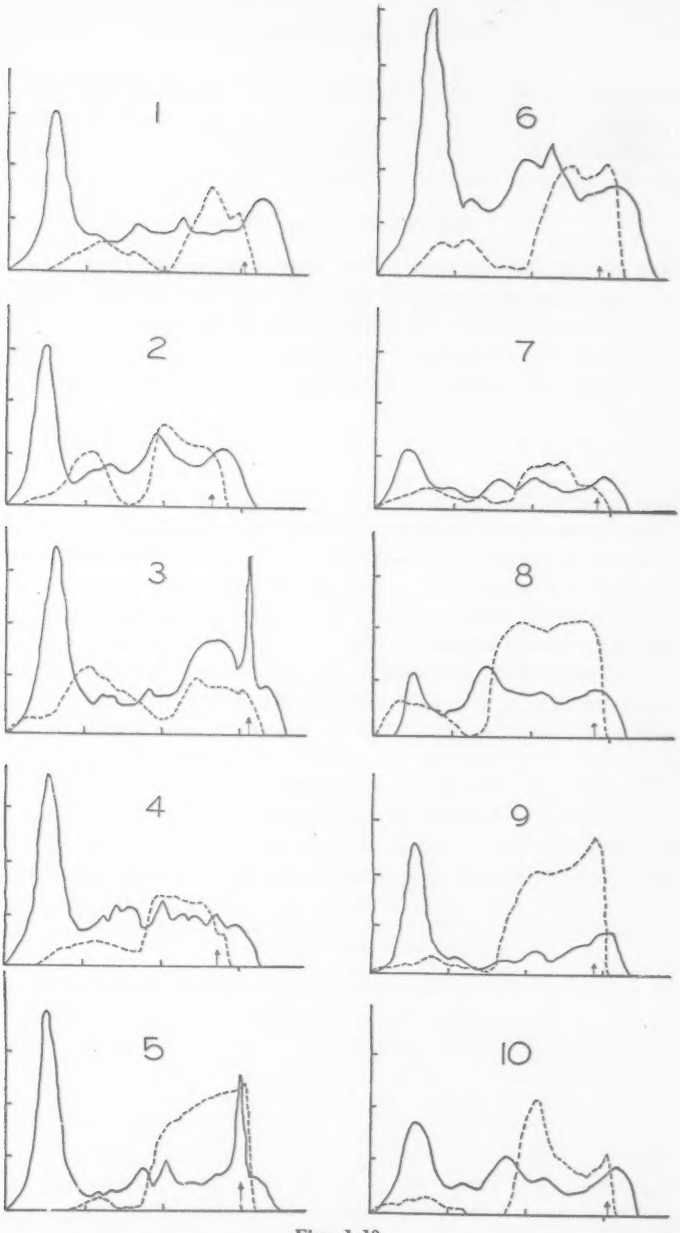
Fig. 5. Electropherogram of rabbit with cholesterol and cortisone (Table 5, No. 62) for explanation see caption to Fig. 1.

Figs. 6 and 7. Electropherogram of myxedema, before and after thyroid treatment (Table 2, 1-A, 1-B). For explanation see caption to Fig. 1.

Fig. 8. Electropherogram of nephrotic stage of glomerulonephritis (Table 3, Case 11). For explanation see caption to Fig. 1.

Fig. 9. Electropherogram of idiopathic hyperlipemia (Table 4, Case 21). For explanation see caption to Fig. 1.

Fig. 10. Electropherogram of idiopathic hypercholesteremia (Table 4, Case 31). For explanation see caption to Fig. 1.



Figs. 1–10

rapidly, followed by those of man, rabbit, and monkey. Globulin patterns were similar in the species investigated except for marked predominance of β-globulin in the dog and separation of both α- and β-globulin in rabbit plasma into multiple components.

Pathologic Conditions in Man

Paper electrophoretic analyses of serum lipoproteins combined with chemical analyses of serum lipid partitions offer an excellent technic for detailed studies of certain pathologic states in man. This holds particularly for conditions associated with disturbances of lipid metabolism such as myxedema, the nephrotic syndrome, idiopathic hyperlipemia, and hypercholesteremia.

Myxedema

Table 2 shows the result in two cases of myxedema before and after treatment with thyroid extract. In Case 1 all serum lipids are increased including the zero fraction, while the α-fraction is reduced. Thyroid therapy resulted in restitution of normal values and profile along with a clinical response (Figs. 6 and 7). Case 2 is less typical, possibly because of complicating cardiovascular disease; the patient later died following a myocardial infarction. Although the concentration of serum phosphatides and cholesterol decreased under thyroid treatment the persistent electrophoretic abnormality might reflect the atherosclerotic condition.

Nephrosis

The considerable elevation of all the lipid fractions in nephrosis is associated with visible lipemia in the first three cases of Table 3 (Fig. 8). The electropherograms show a reduction of the α-fraction and increase of

Table 2. Lipid Partition and Lipoprotein Patterns in Myxedema Patients

before (a) and after (b) thyroid treatment

Case No.	*Total lipids* (mg./100 ml. serum)	*Neutral fat* (mg./100 ml. serum)	*Phosphatides* (mg./100 ml. serum)	*Cholesterol* (mg./100 ml. serum)		*Percentage of sudanophilic lipids*		
				Total	*Est.*	α	β	"0"
1-A	1745	280	620	570	400	20	51	29
1-B	970	280	280	275	200	29	59	12
2-A	900	90	380	290	205	32	54	14
2-B	780	320	210	170	115	16*	57*	27
Normal	860	275	260	215	160	35	52	13

* Decreased mobility.

Table 3. Lipid Partition and Lipoprotein Patterns in Nephrosis

Patient No.	*Diagnosis*	*Total lipids* (mg./100 ml. serum)	*Neutral fat* (mg./100 ml. serum)	*Phosphatides* (mg./100 ml. serum)	*Cholesterol* (mg./100 ml. serum)		*Percentage of sudanophilic lipids*		
					Total	*Est.*	*α*	*β*	"0"
11	Nephrotic stage of nephritis	2920	1205	575	765	545	13[a]	42	45
12	Nephrotic stage of nephritis	4080	1970	850	865	575	6	61	33
13	Nephrosis in lupus erythematosus	2340	1430	370	380	235	8[a]	55	37
14	Kimmelstiel-Wilson syndrome (clear)	1335	425	335	385	280	23	53[a]	24
15	Kimmelstiel-Wilson syndrome (clear)	1445	540	340	390	260	22	49 + 21	8
Normal		860	275	260	215	160	35	52	13

[a] Accelerated mobility.

the "0"-fraction. There is considerable increase of the β-fraction in Cases 12 and 15. A similar behavior may be seen in Patient 13, whose nephrotic syndrome is associated with disseminated lupus erythematosus. In these cases β_1- and β_2-globulin fractions separated.

Patients 14 and 15 illustrate the abnormalities seen in Kimmelstiel-Wilson syndrome, a nephrotic syndrome associated with diabetes. The elevation of the lipids is less striking, but the shifts in the profile occur in the same direction: both cases showed hypoalbuminemia. The fact that the cholesterol rather than the neutral fat is increased in these cases may be responsible for the clearness of their sera. The lactescence of the serum appears to be caused by very high neutral fat concentrations rather than by high cholesterol.

Idiopathic Hyperlipemia and Hypercholesteremia

Idiopathic hyperlipemia is characterized by milky appearance of the serum, caused by the marked increase of neutral fat fraction which is associated to a lesser extent with elevation of other serum lipids. The severe alteration of serum lipids in these patients is not caused by any disease known to produce such changes. This disorder of lipid metabolism is, according to our observations (4, 5), often familial and represents in its severe form a predisposition for advanced premature atherosclerosis.

Table 4. Lipid Partition and Lipoprotein Patterns in Idiopathic Hyperlipemia and Hypercholesteremia

Patient No.	*Total lipids* (mg./100 ml. serum)	*Neutral fat* (mg./100 ml. serum)	*Phosphatides* (mg./100 ml. serum)	*Cholest.* (mg./100 ml. serum)		*Percentage of sudanophilic lipids*		
				Total	*Est.*	α	β	"0"
Idio. Hyperlip.								
21	3580	1735	695	795	515	5	61	34
22	2150	810	490	575	400	13	40 + 10	37
23	2400	1295	500	410	285	15	41 + 14	30
24	3260	1945	590	500	330	9	51	40
25 (diab.)	2885	1900	385	410	295	5	39 + 17	39
Idio. Hypercholest.								
31 (clear)	1375	335	370	450	320	10	71	19
32 (clear)	975	280	285	275	200	6	72	22
Normal	860	275	260	215	160	35	52	13

Milder forms of idiopathic hyperlipemia may remain asymptomatic for many years. The lipoprotein patterns of this entire group invariably show an abnormally low α-fraction and very high concentrations (30–40 per cent) of the "0"-fraction (Table 4, Fig. 9).

Patients 31 and 32 are cases of idiopathic hypercholesteremia. In this condition the serum is clear and shows elevation of the cholesterol and of the phosphatides. The lipoprotein profiles resemble those seen in the previous group in the decrease of the α-fraction; in contrast there is a marked increase in the β-fraction rather than in the "0"-fraction. (Fig. 10)

It may be noted that in the serum of Patient 24 the β-globulin fraction is split and that in Patient 31 α_2-globulin is increased. Elevated γ-globulin was noted in Patient 22, who had a markedly enlarged, probably cirrhotic liver.

Animal Experiments

No. 41 exemplifies the serum of pyridoxine-deficient monkeys.* The lowering of the phosphatides and cholesterol, without changes of the lipoprotein, are atypical and reflect the poor nutritional state of the animals.

Dogs, when rendered myxedematous by large doses of radioactive iodine, show considerable elevation of cholesterol and phosphatides and a corresponding increase of the β-fraction at the expense of α-fraction. Similar changes may be brought about by continued feeding of cholesterol. Administration of cortisone in daily doses of 200 mg. per animal to

* Provided through the courtesy of Dr. Gladys Emerson of Merck Research Institute.

Table 5. Lipid Partition and Lipoprotein Patterns in Induced Abnormal States

	Species	Total lipids (mg./100 ml. serum)	Neutral fat (mg./100 ml. serum)	Phosphatides (mg./100 ml. serum)	Cholest. (mg./100 ml. serum)		Percentage of sudanophilic lipids		
					Total	Est.	α	β	"0"
40	Monkey normal	780	290	280	140	105	41	41	18
41	Monkey B_6 def.	650	455	165	90	55	39	50	11
50	Dog normal	910	315	315	190	135	70	22	8
51	Myxedema	1310	285	500	345	260	52	34	14
52	Cholesterol fed	1010	0	505	335	245	61	31	8
53	Chol. fed and cortisone	2470	610	805	735	465	49	44	7
60	Rabbit normal	440	230	120	60	45	32	50	18
61	Cholesterol fed	2400	250	380	1210	965	2	53	45
62	Chol. fed and cortisone	7020	270	2970	2580	1750	3	97	

cholesterol-fed dogs resulted in additional enormous enhancement of all plasma lipids. The plasma became milky and the α-fraction was reduced to below 50 per cent, a low value for the dog. The changes brought about by cortisone administration confirm our previous observations on the important role played by the adrenal cortex in lipid metabolism (4). (Table 5).

The most striking lipid changes were brought about in the rabbit. One gram of cholesterol was fed to normal rabbits by a technic previously described by Wang, Schaefer, and Adlersberg without the use of a fat vehicle or stomach tube (7). Plasma cholesterol rose to a value twenty times the normal, and the phosphatides and the total lipid to three and five times their normal values. The plasma had the appearance of cream. This was accompanied by extreme changes in the lipid electropherogram: α-lipoprotein was almost absent, while the "0"-fraction rose to 45 per cent. Marked atherosclerosis, as well as cholesterol deposition in skin, joints, and other tissues, was observed. The combination of cortisone injection of 1.25 mg./Kg./day with cholesterol feeding resulted in further elevation of all the lipid levels and in a complete distortion of the lipoprotein profile. As the total lipid content of the plasma in this instance reached or even surpassed the total protein content, it becomes impossible to interpret the electropherogram in the customary terms (Table 5, Fig. 5).

Lipoalbumin

A protein-lipid system which lends itself much better than the above complex mixtures to in vitro studies and model experiments is the system

serum albumin-fatty acids (18). As the latter lend themselves particularly well to the building up technic of Blodgett and Langmuir, we have studied the reaction of such monomolecular layers of stearic acid and its homologs with serum albumin preparations. Since one partner of the reaction, the fatty acid, is present in extremely small quantities, one can get along with very small amounts of albumin, on the average 5 mg. in 30 ml.

An 0.016% albumin solution is placed in a square glass cell; against one of its walls we place the metal slide with a known number of layers of stearic acid. The solution is rapidly stirred and kept at a given temperature. After short intervals of 10 or 20 minutes, the stearic acid plate is removed and its thickness reexamined by monochromatic interferometry or by the ellipsometer. It will invariably be found that a number of layers have been ripped off during exposure to the albumin solution (19). It should be noted that the ripping-off phenomenon is greatly accelerated by phosphate buffer, possibly because of the solubilizing influence of the phosphate upon the albumin-fatty acid complex.

From the physicochemical point of view, it is of interest to observe that the stearic acid film, which consists of a mixture of stearic acid and barium stearate, is not dissolved completely, layer by layer, from the plate. The action of the albumin is rather selective, as it takes off stearic acid and leaves a skeleton of barium stearate behind. This circumstance can not be detected by the optical method which only indicates the overall loss on the plate, but it may be demonstrated by filling out holes, left by the stearic acid, with a suitable substance, whereupon optical methods will show the original thickness to be restored.

Another peculiarity of monolayers is their penetrability to other molecules, due to a lowering of their cohesion, and even their melting point, as compared to the same compound in the three-dimensional state. Thus, the stearic acid film may be covered with as many as ten monolayers of cholesterol, ca. 200 Å thick, which are not attacked by albumin themselves, but which permit the reaction to go on between the stearic acid below and the albumin solution above. Moreover, water molecules will penetrate without difficulty through the cholesterol layers and refill the skeletonized film.

The ripping-off effect is highly specific for both components. Serum globulin, ovalbumin, synthetic polypeptides, etc., leave the stearic acid plate unchanged. On the other hand, built-up plates of cholesterol remain undisturbed by the albumin treatment. Amongst homologous fatty acids, the effect is of the same order of magnitude for palmitic acid. Monolayers of shorter chain fatty acids are difficult or impossible to handle at room temperature. Surprisingly enough, acids with more than 18 carbon atoms,

such as arachidic, behenic, and lignoceric acid, also the terminally branched isoarachidic acid and the unsaturated elaidic and brassidic acids do not react with serum albumin.

One may calculate the molar ratio of stearic acid to albumin when the reaction has reached equilibrium. The values vary for different albumin preparations from 2.3 to 2.7 moles of fatty acids per mole of albumin. This corresponds to about 1.5 per cent by weight of the albumin, figures much smaller than the percentage of mixed lipoids combined with globulin fractions. We have to keep in mind that the original albumin may contain, before the experiment, some fatty acids or related aliphatics. Crystalline Bovine Serum Albumin (Armour) contains approximately one molecule dodecyl alcohol, which has been added to enhance crystallization.

There is a great number of anionic and neutral substances whose reaction with serum albumin has been studied. We should like to mention amongst them a number of carboxylic acids, primarily the lower fatty acids, studied by Murray Luck and his coworkers (20–23). Their combination with serum albumin seems to stabilize the protein against heat coagulation. The adsorption of anionic dyes of varying molecular dimensions has been investigated by Klotz *et al.* (24, 25, 26, 27) who found molecular ratios from about 2:1 to 4:1 in methyl orange:albumin by a diffusion technic. Westphal *et al.* (28, 29) have developed this technic for the study of serum albumin in pathologic and experimental conditions. They report that the dye affinity of the albumin is reduced by the addition of various fatty acids and that this competitive effect is maximal for myristic acid with 14-C-atoms and falls off sharply to reach zero for the homologs above stearic acid. This observation seems to dovetail with ours. Also our findings that the adsorption of azorubin on albumin reduces its affinity for stearic acid, indicates that we are dealing with the same site on the albumin molecule.

We have compared various fractions of human and horse serum albumin, obtained by a variety of methods. Their ability to rip off and dissolve stearic acid parallels their affinity for the dyestuffs. If one extracts the original lipid contained in the albumin and estimates it colorimetrically by Swahn's technic (30), one finds that affinity for stearic acid decreases as the actual lipid content increases.

Whether the lipid content determines the physicochemical behavior of individual albumin fractions or whether inherent differences in chemical constitution of the albumin fractions govern their lipid content, remains to be seen. The heat coagulability of various albumin fractions is undoubtedly a function of their lipid content. On the other hand, deviations from

the normal coagulation point have been correlated with various groups of diseases such as rheumatic and infectious conditions and, in particular, cancer. We are at present studying the individual albumin fractions in patients' sera in respect to their lipid affinity and coagulation temperature.

One of the primary problems in serum lipoprotein research is the correlation of the individual types of lipids with the protein-bound lipid. While we have advanced some ideas as to which lipids are bound to which protein fractions, an accurate quantitative assignment is necessary as basis for the understanding of the reactions and properties of the serum lipoproteins, the nature of the linkages which hold them together, and the character of the agents which break them up—like *Clostridium welchii* toxin—or which act as clearing factors, like heparin.

SUMMARY

The chemical analysis of the lipids in serum or plasma yields data for total lipids, phosphatides, total and esterified cholesterol, and by subtraction, for neutral fat. Zone electrophoresis, for which experimental details are given, shows the distribution of the serum lipids between α-globulin, β-globulin, and a fraction which does not move in the electric field. Values are given for normal man, monkey, dog, and rabbit. Their distinctive patterns are correlated with differences in the propensity of these species to develop atherosclerosis.

Combined studies of the lipid partition and the electrophoretic profile were carried out on patients with disturbed lipid metabolism, particularly myxedema, nephrosis of various etiology, and idiopathic hyperlipemia and hypercholesteremia. The lactescence of lipemic sera is ascribed primarily to increases of the neutral fat and corresponding increases of the electroneutral fraction of the lipoprotein electropherogram. In the Kimmelstiel-Wilson syndrome, the nephrotic syndrome in diabetes, and in idiopathic hypercholesteremia, where the cholesterol level is increased but the neutral fat level normal, the sera usually remain clear.

In animal experiments the destruction of thyroid tissue and, in particular, the feeding of cholesterol induces hypercholesteremia and hyperphosphatidemia in dog and rabbit. The signs of lipemia are further enhanced by the simultaneous administration of cortisone, when the total lipids in the rabbit run sometimes above 7 per cent of the serum. This experimental condition is associated with severe atherosclerotic lesions.

A model for lipoproteins is provided by the reaction between serum albumin and fatty acids, the latter in the form of monomolecular layers.

The technic is described. The specificity of the ripping-off reaction and its parallelism with the dyestuff affinity of serum albumin are illustrated and discussed. The application of the reaction to the study of the "albumin defect" in infectious, rheumatic, and malignant disease is suggested.

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Effects of Diet on Blood Lipids In Man

Particularly Cholesterol and Lipoproteins

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THERE ARE GOOD REASONS for the current great interest in the effects of the diet on the blood lipids. It is now generally agreed that there is an important relationship between the concentration of certain lipid fractions in the blood and the development of atherosclerosis and the coronary heart disease it produces. The outstanding characteristic of atherosclerosis is the presence of lipid deposits, mainly cholesterol, in the walls of the arteries. And both in man and animals the most obvious factor that affects the blood lipids is the diet.

Broadly speaking, as indicated in Table 1, the lipids in the blood are carried in two forms, as emulsions or "chylomicrons," and in the form of complexes with proteins. The present discussion is concerned primarily with the latter, the lipoproteins. These may be separated analytically by fractional precipitation, as in Cohn's alcoholic system; by ultracentrifugation in a medium of known density; by paper electrophoresis; or, with less success so far, by chromatography. Both alcoholic

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Table 1. Primary Forms of Serum Lipids and Approximate Distribution, in Healthy Persons, of Lipoprotein Lipid (LPL), and Total Serum Cholesterol (Chol.) in These Forms

Class	*Distribution*	
	LPL (%)	*Chol.* (%)
A. Emulsions ("chylomicrons")	0	0
B. Solutions (lipoproteins)	100	100
1. α lipoproteins	20–30	20–30
2. β lipoproteins[a]	70–80	70–80

[a] Includes Gofman's "giant molecules."

precipitation and paper electrophoresis reveal two distinct fractions, the alpha lipoproteins and the larger beta lipoproteins which are more readily precipitated and move more sluggishly in an electrical field. Subfractions of these two classes are less sharply differentiated and their significance is more obscure. But all of the lipoproteins are notable in that they contain cholesterol and phospholipids, apparently as essential ingredients, as well as proteins and other lipids. The main interest in regard to lipids in atherogenesis is centered on the cholesterol and on the beta lipoproteins which contain most of this cholesterol.

Quantitative analysis of the lipoprotein system in the blood is technically not simple but progress in methods has been rapid recently. It has become increasingly easy to carry out controlled dietary experiments on animals so as to study the effects on the various items of the lipoprotein system. Unfortunately, different animal species differ markedly and in none of the species studied is there a close quantitative resemblance to man, either in regard to the effects of the diet on the blood or in regard to the tendency to deposit cholesterol in the coronary arteries.

This is most easily shown, as in Table 2, by the results of cholesterol-feeding experiments. The rabbit and the chick are at one end of the scale of sensitivity to exogenous cholesterol while man is far removed at the other. The addition to the diet of an amount of cholesterol that produces a rise in the serum cholesterol in the rabbit of 1000 per cent or more, produces in man either no change or perhaps a 10 per cent rise when the cholesterol load is consumed for months on end.

Accordingly, if it is desired to discover quantitative relationships in man, it is necessary to study man himself. But controlled human dietary experiments are difficult and costly. Many individuals must be studied in prolonged experiments because the significant time scale is long and individual variability is large. Finally, there is always the question as to what may be the influence of the previous lifetime of dietary habitus.

Table 2. Effect, in Various Species, on the Concentration of Total Cholesterol in the Blood Serum of Addition of Cholesterol to the Diet

Species	*References*	*Cholesterol*		
		Diet (mg./Cal.)	*Weeks*	*Serum* (% increase)[a]
Rabbit	37–43	0.4–5.0	5–16	200–3000
Chick	44–46	0.8–6.0	5–25	50–600
Rat	37, 47–49	3–30	4–22	50–200
Guinea pig	37	5	5	180
Dog	50, 51	2	1–2	100
Monkey	52, 53	3	29	22
Child	54	0.4–1.0	1–3	0
Woman	55, 56	1.0	16	0
Woman	57	1.4[b]	4	8
Man	58	1.5	6	"Slight"
Man	59	2[b]	6–7	11–18
Man	59	16[c]	4	3
Man	59	16[c]	9	5

[a] As a percentage of the initial (control) level.
[b] As egg yolks.
[c] As egg yolk powder.

Nevertheless, there is a growing accumulation of knowledge from controlled dietary experiments on man. Some of this will be presented here but the main emphasis will be on data obtained from samples of human populations habitually subsisting on different diets.

METHODS

The serum cholesterol values cited here were obtained by the method of Abell *et al.* (1) ("Kendall method") which involves saponification prior to the eventual estimation with the Liebermann-Burchard reagent. This method has checked repeatedly, with fully satisfactory agreement, with the method involving precipitation with digitonin (2, 3). A systematic comparison of 134 serum samples, in quadruplicate, analyzed by the Kendall method and by the ordinary application of the Liebermann-Burchard reagent to the Bloor extract, as previously used by us (4, 5–8), gave the prediction equation: $Y = 0.836X$, where Y is the Kendall method result and X is the result with the Bloor extract method. The standard error of estimate in this prediction proved to be ± 10.23 mg. per 100 ml.

Ultracentrifugal analysis was made in two stages by the flotation method of Gofman (9), with modifications as recommended by Dr. Gofman and other experts in the U.S.P.H.S. Cooperative Study.

Cholesterol in the alpha and beta lipoprotein fraction of the serum was analyzed in the fractions separated by a modification of Cohn's Method X which uses low-temperature precipitation in alcoholic solution.

The amount of lipid in the alpha and beta lipoproteins and in the chylomicrons was estimated by Swahn's (10) paper electrophoresis method; the lipid is measured by the amount of Bismarck or Sudan Black adsorbed on the paper after electrophoresis.

Relative obesity was estimated in terms of relative body weight and of the thickness of the skinfolds. Relative body weights are expressed as percentages of the average weight for given height and age as given in the tables of the Medico-Actuarial Investigations on Mortality of 1912; these are sometimes referred to as Davenport's Height-Weight Tables. Skinfold thickness, comprising a double layer of skin and subcutaneous fat, was measured with skinfold calipers at constant tension (11).

SUBJECTS

Most of the data to be cited here were obtained from the groups indicated in Table 3, all of whom were physically healthy men between the ages of 18 and 65. The experimental subjects were schizophrenic patients found to be metabolically "normal" on careful study. During the experiments on them they were separately housed and fed in a special diet kitchen and were under rigid 24-hour control, both as to diet and

Table 3. PRINCIPAL GROUPS OF MEN REPRESENTED IN DIET AND BLOOD LIPID STUDIES CONSIDERED IN THIS PAPER

1. Minnesotans	(Business and professional men)
2. Experimental subjects	(Hastings State Hospital Patients)
3. U. S. Army	(Enlisted men in the United States)
4. Neapolitan clerks	(Naples Tax Offices)
5. Neapolitan firemen	(Naples Fire Department)
6. Neapolitan heavy workers	(ILVA Steel Works)
7. Neapolitan "bankers"	(Naples Rotary Club)
8. Bologna policemen	(Bologna Police Department)
9. Swedish clerks	(Kochums Shipyard, Malmö)
10. Swedish firemen	(Malmö Fire Department)
11. Swedish heavy workers	(Kochums Shipyard, Malmö)
12. South African Bantu	(Johannesburg)
13. South African Europeans	(Johannesburg)
14. Spanish lower class	(Madrid)
15. Spanish professional men	(Madrid)
16. Englishmen	(London, light industry)

as to physical activity. Finally, measurements were made on ambulatory patients who had definite coronary disease as indicated by previous myocardial infarction and/or angina pectoris and electrocardiographic changes.

INTERRELATIONS BETWEEN LIPID FRACTIONS

Cholesterol and Phospholipid

There is a general tendency for some of the lipid fractions to be interrelated so it might be expected that the measurement of the response of one fraction to the diet would allow the prediction of changes in other fractions. This assumption is not tenable, however, even in clinically healthy men and with such closely related items as cholesterol and phospholipid. For example, in blood serum samples from 59 healthy young men we found a correlation coefficient of $r = 0.847$ between total cholesterol and phospholipid. This is very impressive but even so it does not allow accurate prediction of one from the measurement of the other item. Incidentally, the ratio of cholesterol to phospholipid in the serum has been advocated frequently as a useful device to detect men who have or may be threatened with coronary heart disease.

Alpha Lipoprotein and Chylomicron Lipids

As shown in Table 4, the amount of lipid in the lipoproteins is very largely independent of the amount of lipid in the emulsion or "chylomicron" form in the serum. In healthy men there was no correlation at all between alpha lipoprotein lipid and chylomicron lipid concentrations. The small correlation indicated between beta lipoprotein lipid and chylomicrons could be an artifact owing to the uncertainty of separation of a portion of these fractions in paper electrophoresis. Finally, the concentration of total cholesterol in the serum shows only a trivial relationship with the concentration of chylomicron lipid in the same serum.

Table 4. Correlation Coefficients between the Concentration of Chylomicron Lipid and the Lipid in Alpha and Beta Lipoproteins the Total Cholesterol Concentration in Blood Serum from 247 Clinically Healthy Men in Naples, Italy

No.	*Age*	*Lipoprotein lipid*		*Cholesterol*
		α	*β*	
104	18–34	−0.12	0.28	0.15
143	35–50	0.00	0.41	0.20

Table 5. Correlation Coefficients of Total Cholesterol with Alpha Lipoprotein Lipid ($r_{c,\alpha}$) and with Beta Lipoprotein Lipid ($r_{c,\beta}$) in Clinically Healthy Men in Naples, Italy

No.	Category	Correlation coefficient	
		$r_{c,\alpha}$	$r_{c,\beta}$
119	Heavy workers	0.04	0.69
150	Firemen	0.03	0.63
26	Clerks	$\cong 0$	0.69
26	Bankers	$\cong 0$	0.60

Beta Lipoprotein and Chylomicron Lipids

The total cholesterol concentration in the serum shows no correlation with the amount of lipid in the alpha lipoprotein fraction but it is highly correlated with the amount of lipid in the beta fraction. Table 5 shows the results of serum analyses for 321 men in Naples. It will be observed that the correlations are substantially identical in all four categories of men, in spite of their differences in physical activity and the marked difference of the diet of the "Bankers" from the diets of the other men. Since only single analyses for lipoprotein lipids were available and there are appreciable method errors involved in the cholesterol method and particularly in the paper electrophoresis method, the true correlation between total cholesterol and beta lipoprotein lipid in these men is higher and is estimated to be of the order of $r = 0.7$–0.8.

Gofman (12) has insisted that there is very little correlation between the total serum cholesterol concentration and that of his S_f 12–20 fraction separated in the ultracentrifuge. Actually, the correlation is always significant and in many series is of the order of $r = 0.6$ (13, 14).

Alpha and Beta Lipoprotein Lipids

Among the cholesterol fractions in the serum it is obvious that there must be a very high correlation between the total cholesterol and the cholesterol in the beta lipoprotein since the latter contains some three quarters of the former. In a series of 54 healthy men aged 50–60 years, we found four fifths of the total cholesterol was in the beta lipoprotein fraction and in a series of 54 men of the same age with coronary disease, the beta lipoprotein fraction accounted for an average of 87.5 per cent of the total cholesterol. However, there is almost complete independence between the amounts of cholesterol in the alpha and in the beta lipoproteins. In the two series noted above the correlation coefficients between total cholesterol and alpha lipoprotein cholesterol were, respectively, $r = 0.063$ and $r = 0.195$.

All of the foregoing indicates that the effects of the diet on each of the several blood lipids should be separately considered though, as will appear, there are important common trends in the responses.

DIETARY CHOLESTEROL

The lack of effect on the blood cholesterol of man of adding large amounts of cholesterol to the diet has already been mentioned (cf. Table 2). Population surveys (4, 15, 16) have confirmed the lack of response in man to the cholesterol level of the diet so this point requires no further consideration. Where serum cholesterol differences have been found to be associated with differences in dietary cholesterol intake, other dietary differences have been involved (17, 18).

EFFECTS OF CALORIES AND RELATIVE OBESITY

The "calorie habitus" of a man may be inferred from its result as seen in his relative emaciation or obesity. And, in turn, the relationship between the calorie habitus and the serum lipids may be inferred by comparing the serum lipids of men of differing relative obesity, provided other factors, such as age and occupation, are constant. Five years ago we reported (4) that among sedentary businessmen aged 48–55 in Minnesota, the fatter men tended to have very slightly higher serum total cholesterol concentrations than their thinner counterparts. A similar slight tendency is observed among Danes when they are classed into "leptosomatic" and "pyknic" types (19). A more significant relationship in 46 young Englishmen, with a correlation coefficient of $r = 0.43$, between total serum cholesterol and subcutaneous fat thickness, was reported by Tanner (20). Among both men and women of the lower class of Madrid, Spain, and among men of the working class of Naples, Italy, we found significantly higher serum total cholesterol concentrations in the fatter persons than in their thinner counterparts (5, 8, 16, 21, 22). On the other hand, among men of the professional class in Madrid and among employees in light industry at Slough, near London, the tendency for a relationship between relative obesity and serum total cholesterol was slight, like that seen in Minnesota, and proved to be statistically not significant (6). These studies indicated that there is no general obligatory relationship between relative obesity and the serum total cholesterol concentration.

In the past six months it has been possible to collect more extensive data on the relationship between obesity and serum lipids. Table 6 summarizes data from heavy workers, firemen, and clerks in Naples, Italy. The concentration of lipid in the serum alpha lipoprotein fraction was

Table 6. Coefficients of Correlation for Relative Body Weight with the Concentrations in the Blood Serum of Total Cholesterol, of Alpha Lipoprotein Lipid and with Beta Lipoprotein Lipid in Healthy Men in Naples

No.	*Age*	*Occupation*	*Mean rel. body wt. (%)*	*Correlation with:*		
				Chol.	*α*	*β*
55	19–34	Heavy labor	98.7	0.35	−0.07	0.39
49	19–34	Firemen	103.8	−0.13	−0.07	0.15
22	19–34	Clerks	96.9	0.44	..	..
64	35–54	Heavy labor	97.7	0.41	0.12	0.38
101	35–54	Firemen	101.7	0.33	0.09	0.32
37	35–54	Clerks	97.4	0.46	..	..

Table 7. Fatness and Serum Cholesterol

Healthy Men of the Working Class in Naples, Segregated for Fatness by Skinfold Thickness (Arm + Scapula)

No.	*Skinfold (mm.)*		*Cholesterol (mg./100 ml.)*	
	Range	*Mean*	*Mean*	*S. E.*
64	8–23	15.8	151.7	±3.4
64	23–83	36.6	180.8	±4.0
Difference			29.1	±5.2

completely independent of relative body weight both in younger (19–34 years) and older (35–54 years) men. But significant correlations with relative body weight were found for both total serum cholesterol and for the lipid in the beta lipoprotein fraction.

Relative body weight is not an exact measure of true obesity or fatness, and skinfold thickness, which is a direct measure of subcutaneous fat, may be more satisfactory. As shown in Table 7, skinfold thickness and serum total cholesterol are also correlated in these Neapolitans of the working class so there is no doubt about the effect of obesity in this population.

In contrast to the Neapolitans, Table 8 summarizes a simultaneous study on 134 clinically healthy Minnesotans. In these men relative obesity is not related to the serum cholesterol concentration. Only one point of consistency seems to emerge so far. In populations where the general level of cholesterol in the blood is low, as in Naples (5) or in poor men in Madrid (8), the cholesterol concentration tends to be correlated with relative obesity. And in populations characterized by high serum cholesterol, as in England (6), Minnesota, and among rich people in Madrid (8), there is little relationship to obesity.

Table 8. Relative Body Weight vs. Serum Total Cholesterol Concentration, in 134 Clinically Healthy Men, Aged 50–62, in Minnesota

No.	*Relative body weight* (Kg.)		*Cholesterol* (mg./100 ml.)	
	Range	*Mean*	*Mean*	*S. E.*
51	<95	86.9	222.7	±4.7
44	95–104.9	100.4	228.0	±5.9
39	>105	112.7	225.1	±6.6

Table 9. Serum Cholesterol Concentration and Weight Gain from Simple Overeating
Means and Standard Errors for 20 Men

Period	*Weight* (Kg.)	*Cholesterol* (mg./100 ml.)
Control	68.4 ± 2.2	228.5 ± 7.4
5 weeks	71.1 ± 2.3	248.8 ± 9.1
20 weeks	78.9 ± 3.1	252.2 ± 12.7

This is the situation when we study men who are constantly thin or fat in a stable situation. Among men who are losing weight and are actively underfed, the serum cholesterol is almost always low (22, 23, 24). And, as shown in Table 9, among men who are actively gaining weight from overeating, the serum cholesterol tends to rise when weight gain starts but to increase little thereafter as weight gain continues.

This is observed in controlled experiments where the diet is not changed in kind but is merely eaten in excess so that from a few to over 20 Kg. weight is gained in six months. A similar serum cholesterol response is seen in populations who gain weight after the privations of war (23, 24). And it may be remarked that in such populations there is also a parallel change in the incidence and mortality from arteriosclerotic heart disease.

CHARACTER OF THE DIET: HUMAN EXPERIMENTS

It is now well known that when patients are put on extremely low fat diets, such as Kempner's rice-fruit diet, there usually is a prompt and substantial fall in the blood cholesterol (7, 25–28). Some years ago we showed that the absence of cholesterol in the diet is not responsible but that any diet low in fat tends to have this effect (7, 13). During the past four years we have studied this question systematically and Table 10 summarizes some of the results.

Table 10. Change in Serum Lipid Concentrations in Physically Healthy Men, as Percentages of the Control Values, after 3 to 4 Weeks on Experimental Diets

Mean Values, and Standard Errors, for Total Cholesterol, S_f 12–20 Lipoprotein, α and β Lipoprotein Cholesterol, and Phospholipids

Item	*% calories from fats*		
	24	*18*	*9*
Cholesterol	−7.6 ± 2.0	−8.7 ± 2.4	−9.8 ± 2.2
S_f 12–20	−8.4 ± 5.4	+12.2 ± 17.5	−0.3 ± 5.2
α Cholesterol	−8.2 ± 2.0	..	−5.3 ± 2.7
β Cholesterol	−6.0 ± 3.0	..	−10.8 ± 3.4
Phospholipid	+1.8 ± 3.4	..	−6.7 ± 4.2

The control diet provided 38 to 39 per cent of calories from fats. Thirty-five to 48 men in each experiment except on the 18 per cent fat diet where there were 23 men.

Table 11. Change in Serum Cholesterol from Control Level after Change to a Moderately Low-fat Diet Rigidly Constant for 32 Weeks

Means for 9 Men

Weeks	*Serum cholesterol change* (mg./100 ml.)	*Weeks*	*Serum cholesterol change* (mg./100 ml.)
1	−17	16	−31
2	−16	28	−28
4	−24	32	−26

In these experiments each subject was stabilized for at least a month on the control diet and the intake of calories, proteins, vitamins, and cholesterol was constant throughout. The only alteration was the substitution of carbohydrate calories for fat calories. This dietary manipulation clearly resulted in a change in the serum concentration of total cholesterol and of the cholesterol in both the alpha and beta lipoprotein fractions. The changes in the S_f 12–20 ultracentrifugal fraction and in the phospholipids, however, were not statistically significant.

Such experiments are difficult to continue for long but, as shown in Table 11, it was possible to carry out one experiment for 36 weeks, including 32 weeks on the reduced fat diet. The serum lipid responses to such dietary changes persisted as long as we were able to maintain the changed diet.

This is borne out also by the data summarized in Table 12, from the experiment of Groen *et al.* (18) on 60 normal human volunteers. During twelve weeks on each controlled diet there was a major change in serum cholesterol in the first month and subsequently a slight tendency for the change to increase as the diet was continued for the next

Table 12. Mean Serum Total Cholesterol Values for 60 Healthy Volunteers Before and at Intervals during Subsistence on a Luxurious Diet and on a Vegetarian Diet

Diet	*Mean serum total cholesterol values*		
	Control	*During diets* (weeks from start)	
		3, 4, 5	*10, 11, 12*
Luxurious	216	242	248
Vegetarian	224	202	199

Data from Groen *et al.* (18).

two months. It is not possible to identify the responsible dietary factor or factors in this Dutch experiment because there were simultaneous changes in fats, calories, proteins, and cholesterol in the diet, but it is clear that a more luxurious high-fat diet caused the cholesterol to rise and a vegetarian diet caused it to fall. On the luxurious diet 45 per cent of the calories were supplied as fats while the corresponding figure for the vegetarian diet was 37 per cent.

POPULATIONS ON DIFFERENT DIETS

We have repeatedly stressed the value of the study of properly selected populations for comparisons of dietary effects as they actually occur in nature (13, 16, 21, 22, 29). If the interest is on diets differing in fat content, then there is a wide range of choice, as indicated in Table 13. Fats provide from less than 10 per cent of the calories, as in Japan, among the South African Bantu, and in many parts of Asia and Africa, to our own level of over 40 per cent in the United States. Most Eskimos have much lower fat diets (30). Swedes and Canadians eat relatively

Table 13. Approximate Percentage of Dietary Calories Supplied from Total Fats in the Period 1951–1953

Country	*Calories from fat (%)*	*Country*	*Calories from fat (%)*
Japan	8	Greece	24
S. A. (Bantu)	10	France	30
India	13	Holland	34
Turkey	14	England	35
Brazil	18	Australia	38
Portugal	20	Sweden	38
Italy	20	Canada	38
Spain	22	U. S. A.	41

Compiled from data of F. A. O. and other sources.

high fat diets, while Spaniards and Italians, on the average, have diets low in fat. It is interesting that in the Western countries, where racial, cultural, and climatic differences are minimal, the range of fat consumption is great but the proportion of calories supplied by proteins is remarkably constant, being consistently from 11 to 14 per cent, usually between 12 and 13 per cent.

From our dietary experiments in Minnesota we expected that differences in fat consumption of populations should be reflected in the serum concentrations of cholesterol and perhaps other components of the lipid system. Explorations in 1951–1952 demonstrated both the feasibility of field work on these questions and the general soundness of the approach (5, 6, 8). In 1954 more extensive studies were possible in Italy and in Sweden. Some of the most recent results are summarized here.

In Naples, where the general diet averages about 20 per cent of calories from fats, we studied 356 heavy workers, firemen, and clerks and 46 men of the wealthy class who eat a diet higher in fats than the general population. As a sample of Italians on a diet much higher in fats than that of Naples or Italy as a whole, we studied 54 policemen of Bologna. The average diet in Bologna is estimated to provide 28 to 30 per cent of the calories from fats. Finally, we studied, simultaneously, 205 men in the Twin Cities of Minnesota in whose diets fats provided slightly over 40 per cent of the calories. The men in the various groups were suitably distributed from age 18 to age 60 and all were clinically healthy. The cholesterol findings are summarized in Fig. 1.

Clearly, the men of the general Naples population have much lower serum cholesterol values than the Minnesotans, the difference of some 40 mg. in the twenties rising to a difference of nearly 60 mg. per cent in the fifties. All of the groups of men show an age trend but this is trivial among the Neapolitan working men after the early thirties. The Naples "bankers" and the Bolognese policemen are intermediate, about as would be predicted on the theory of the controlling effect of the proportion of fat in the diet.

It should be observed that the cholesterol differences summarized in Fig. 1 are considerably greater than we have found in controlled experiments on man with similar diets. This suggests, as is reasonable, that lifelong subsistence on these diets produces more effect than results in a few weeks or months. However, consideration should be given to other factors in the diet or in the mode of life of possible relevance. There are several obvious questions.

First, are there differences in calorie habitus and relative obesity which might be involved? The answer to this is no. The average relative body

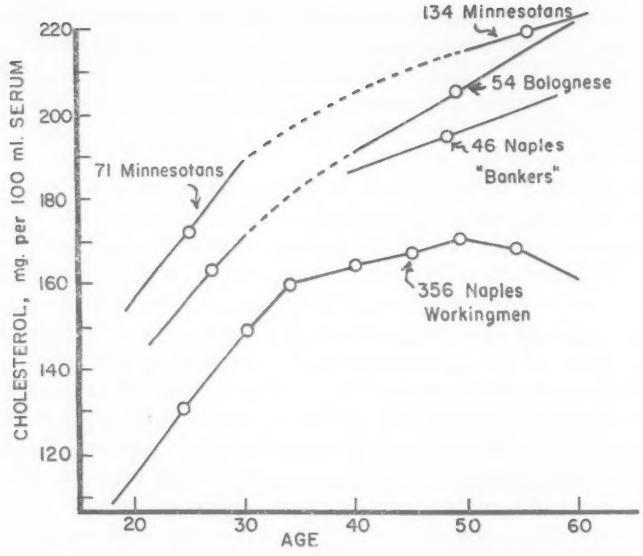

Fig. 1. Serum cholesterol data obtained in 1954 in Minnesota and in Italy. Mean values.

weights of the Neapolitans and of the Minnesotans are both in the range of 98 to 102 per cent of U. S. standards and matching the groups exactly in regard to relative body weight does not affect the observed cholesterol differences. The Bolognese policemen tended to be overweight, averaging 108 per cent for their height and age but, as shown in Table 14, this does not explain the large difference between them and the Neapolitans. A difference of 31.8 mg. per 100 ml., or 18 per cent of the Naples average, remains.

Considerations of differences in physical activity is not helpful either. The physical activity of the Naples firemen is much like that of the Bologna policemen and the physical activity of our Minnesota subjects resembles that of the Naples clerks. And among the Neapolitans, there were no significant cholesterol or other lipid differences related to physical activity; heavy workers, moderate workers, and sedentary clerks were substantially the same.

The factor of race does not seem to be significant; at least it does not explain the serum lipid differences observed between groups of Spaniards (8) or between the various groups of Italians.

It is conceivable that other dietary differences besides those in total fat consumption may affect the serum lipids. In all of the various popu-

Table 14. Serum Cholesterol Concentrations in Bolognese and in Neapolitans Matched for Age and Relative Body Weight

No.	City	Mean age	Mean rel. wt. (Kg.)	Cholesterol (mg./100 ml.)	
				Mean	S.E.
34	Bologna	49.3	108.4	209.3	
34	Naples	49.1	107.9	177.5	
Difference, 34 pairs				31.8	±7.3

lations studied here the proportion of the total calories supplied from proteins is substantially constant and in none of the subjects were there evidences of vitamin or mineral deficiencies. Further and more detailed studies on the diets are desirable but only the factor of fat intake appears important so far.

Studies on Bantu

As a follow-up of our first (1952) report on studies in Italy, Walker and Arvidsson (31) made a study on populations in the Johannesburg area of South Africa. A part of their findings, with the cholesterol values converted to equivalence of the method used here, is summarized in Table 15. The South African Europeans, whose diet follows the North European and American pattern of high fat, are similar in cholesterol concentration to Minnesotans, but the Bantu, whose diet provides only some 10 to 20 per cent of calories from fats, have much lower values. Europeanized Bantu, whose diets are slightly more luxurious than that of their fellows, tend to have slightly higher cholesterol values.

Table 15. Mean Serum Cholesterol Values of Bantu and of Minnesotans (1953–1954), with Equivalent Methods and at Equal Age, Expressed as Percentage of the Mean for South African Europeans (S. A. E.) at Age 28

Group	Mean age 28		Mean age 46	
	No.	Chol. (mg./100 ml.)	No.	Chol. (mg./100 ml.)
S. A. E.	55	100	46	116
Minnesota	70	106	46	125
Bantu	85	81	54	87
Bantu, Eur.[a]	69	86	..	..

Computed from data of Walker and Arvidsson (31) on ordinary Bantu, Europeanized Bantu (Eur.), and S. A. E., and new data on Minnesotans.

[a] Mean age 31 years.

The findings in South Africa fit nicely into the picture of a dominating influence of dietary fats on the serum cholesterol.

Studies in Sweden

Immediately after the end of the work in Italy last spring, parallel studies on men in Malmö, Sweden, were undertaken by Professor H. Malmros and Drs. Gunnar Biörck and Bengt Swahn (32), who had participated in some of the work in Italy. A comparison of some of the findings in Sweden with those obtained in Naples is shown in Fig. 2. There were 459 men in the Swedish series, equally divided among heavy workers, firemen, and clerks.

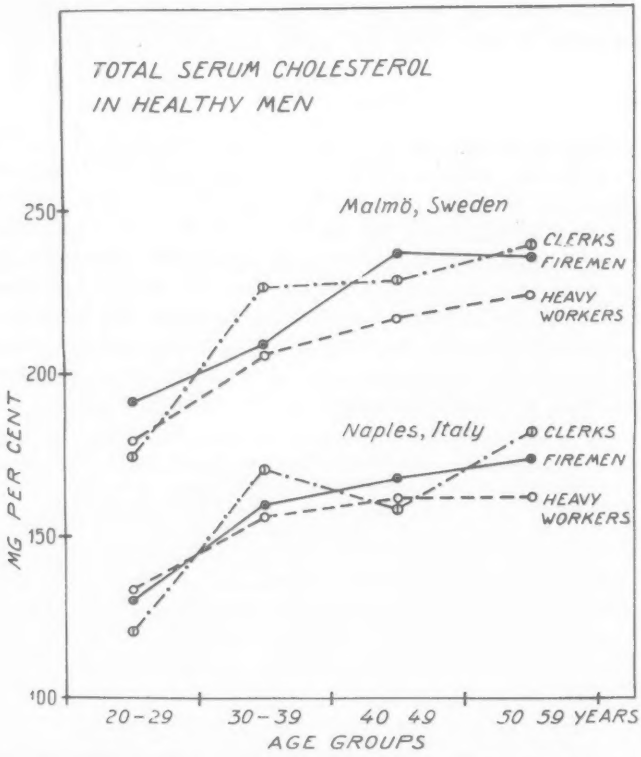

Fig. 2. Comparison of 1954 cholesterol findings in Naples, Italy, and in Malmö, Sweden, the latter being obtained by Prof. H. Malmros and Drs. Gunnar Biörck and Bengt Swahn.

As in Naples, there was little difference between the men according to their physical activity, though there may be a slight tendency for the heavy workers to have lower cholesterol values, especially at the older ages. But the difference between these Swedes and the Neapolitans is very impressive, averaging 50 to 60 mg. per 100 ml. There was a similar but not quite so striking difference between the concentrations of lipid in the beta lipoproteins when the Swedes and the Neapolitans were compared.

There are no reliable figures on the local diet in Malmö but there is no doubt that the fat content there is at least as high, and probably is higher, than the average for the whole of Sweden. For 1953 the average Swedish diet was estimated from official figures to provide 37.5 per cent of the calories from fats and 11.5 per cent from proteins.

Figure 2 compares the approximate average cholesterol concentrations in the serum in several populations of healthy men of age 25 and at age 50. At age 25, the highest averages, in Minnesota and in Southern Sweden, are some 50 mg. of cholesterol per 100 ml. above the averages for Naples and for the Bantu; at age 50 the maximum difference between averages is about 75 mg. per 100 ml. The regularity of the relationship is notable.

Other Studies

The conclusions reached here about the important effect of the level of total fat metabolism on the blood cholesterol in man are supported by other observations. The cholesterol and the beta lipoprotein concentration is low in the serum of Guatemala Indians, who live on a low-fat diet (33, 34). After the war the Germans, who were forced to live on a low-fat diet, had low serum cholesterol values and these rose as the dietary fat was increased (23, 24). Patients put on low-fat, low-cholesterol diets show declines in serum cholesterol and in the beta lipoprotein S_f 12–20 fractions (12).

There are also some questions that need further investigation. It has been reported that heavy drinkers have low serum cholesterol values (35). And it has been reported that artificial diets extremely high in fats, with fats contributing 70 per cent or more of the calories, may actually lower the blood cholesterol (36). What may be involved in these situations is obscure.

Neither space nor presently available knowledge allow a detailed discussion of the mechanisms involved in this dietary fat control of the blood lipids. They are unquestionably very complex.

SUMMARY

1. Lipids exist in the blood serum as emulsions ("chylomicrons") and as solutions of lipoproteins containing proteins, cholesterol, and other lipids. Interest in relation to atherosclerosis is centered on the cholesterol and the beta lipoproteins which contain most of the cholesterol.

2. The diet influences the blood lipids in man and animals but great quantitative differences between species makes it essential to study man himself to discover the effects of the diet on the blood lipids in man.

3. The amount of lipid in the chylomicron form in the serum is practically independent of the concentration of cholesterol and lipoproteins in the serum. The correlation between the concentrations of alpha and beta lipoproteins is very low.

4. Experiments on man show that dietary cholesterol per se, even in large amount, has no important influence on the serum cholesterol concentration but that the latter is markedly affected by the total fat content of the diet.

5. Calorie intake per se has little influence on the blood lipids. Such effect as it has may be secondary to fat metabolism differences commonly associated with differences in calorie intakes.

6. Studies on population samples of healthy men show a marked direct relationship between the content or proportion of fat in the diet on the one hand and the concentration of total cholesterol and of beta lipoproteins in the serum on the other. The effect of the diet tends to be greater in middle-aged men than in younger men.

7. The average concentration of cholesterol in the serum of men in areas where the diets are very high in fats (of the order of 40 per cent of calories), is 25 to 50 per cent greater than the average in areas where the diets are low in fats (of the order of 20 per cent, or less, of calories).

8. A significant effect of dietary fat level on the serum cholesterol concentration is evident in man in a few weeks on a changed diet. The effect tends to increase very slowly with time thereafter and it is most pronounced when comparison is made between populations habitually subsisting on different diets.

9. It is not known how the dietary fat intake exerts its controlling influence on the blood lipids of man but the mechanisms must be complex.

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Oxidation and Synthesis of Fatty Acids in Soluble Enzyme Systems of Animal Tissues

David E. Green

During the past two years the complete reconstruction of fatty acid oxidation with combinations of highly purified enzymes has been successfully accomplished. Now that the excitement of the participating groups has died down it may be desirable to review the general field of fatty acid oxidation and synthesis according to a logical plan without stressing unduly the chronology of developments.

To the best of our knowledge fatty acids are oxidized exclusively within one site in animal cells, the mitochondrion (1, 2). When we say that the process of fatty acid oxidation is localized within the mitochondrion just what do we mean? Do we infer that the mitochondrion is like a red blood corpuscle—a structure with a membrane separating the internal fluid content from the outside medium—and that within the internal fluid all the enzymes and coenzymes of the fatty acid oxidation system are in solution reacting with one another by a process of random collision? Or are we to think of the mitochondrion in terms of the concept of the cyclophorase complex of enzymes (3)—a giant macromolecule arising from the complexing and association of hundreds of separate enzymes—arranged in a very precise and intricate pattern of organization? According to this concept juxtaposition of the enzymes which follow one another serially in a particular metabolic sequence is the principle which makes random diffusion and collision unnecessary, and which makes possible interactions that are difficult to duplicate with combinations of separate, unassociated enzymes. In the closing part of the present review I intend to mention some recent work in our laboratory on one of the enzymes of the fatty acid oxidation system

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Table 1. Products of Oxidation of Fatty Acids

Source of mitochondria	*Even-numbered acids* (C_4 *to* C_{18})	*Odd-numbered acids* (C_5 *to* C_{17})
Heart	CO_2, H_2O	Propionate, CO_2, H_2O
Kidney	CO_2, H_2O	Propionate, CO_2, H_2O
Liver	Acetoacetate, CO_2, H_2O	Acetoacetate, CO_2, H_2O

that may have considerable bearing on the questions which I have just raised.

Let us begin by examining some of the characteristics of fatty acid oxidation as it is catalyzed by suspensions of mitochondria. In presence of kidney or heart mitochondria and with appropriate additions even numbered fatty acids from C_4 to C_{18} (Table 1) are oxidized completely to CO_2 and H_2O (4). The corresponding β-hydroxy acids, α-β unsaturated acids, and β-ketoacids are also oxidized to completion and at about the same rate. The odd numbered fatty acids are oxidized as rapidly as the even numbered fatty acids but with the difference that propionic acid accumulates as an end product in addition to CO_2 and water. When liver mitochondria are used instead of kidney or heart mitochondria, two important differences emerge in the pattern of fatty acid oxidation. In the first place, acetoacetate also accumulates as one of the end products of the oxidation of even numbered fatty acids (4–6). Second, propionic acid does not accumulate as the end product of the oxidation of odd numbered fatty acids since propionic acid is readily oxidized, at least by rabbit liver mitochondria, to CO_2 and water (7).[1]

THE CITRIC ACID CYCLE

Fatty acids as such are not oxidized by mitochondria (8). Unless some member of the citric acid cycle is undergoing oxidation simultaneously fatty acid oxidation does not start (Table 2). Members of the citric acid cycle or substances which give rise to members of the cycle are referred to as sparkers since their oxidation sparks the oxidation of fatty acids. There are two functions served by the sparker which we will consider separately.

First, when any member of the citric acid cycle undergoes oxidation by molecular oxygen, inorganic phosphate in the suspending medium becomes esterified and ultimately converted to adenosine triphosphate (ATP) (9). It is the generation of ATP by oxidative phosphorylation which is one of the two functions of the sparker. ATP triggers the first

[1] Rat liver mitochondria do not show this capacity for oxidizing propionate (5).

Table 2. Sparking of Fatty Acid Oxidation by Fumarate Kidney Cyclophorase System

Additions	*Oxygen uptake*
Butyrate (30 μm)	10
Butyrate + fumarate (0.5 μm)	200
Butyrate + fumarate (3.0 μm)	287
Octanoate (5 μm)	0
Octanoate + fumarate (0.5 μm)	140

The values for the sparked oxidations have been corrected for the oxygen uptake due to the oxidation of fumarate (8).

step of fatty acid oxidation, the conversion of the fatty acid to the corresponding coenzyme A derivative (10–12). Coenzyme A (CoA) is a complex nucleotide whose structure (shown in Fig. 1) has recently been established by Lipmann and his group (13, 14). For present purposes it can be conceived of as merely a vehicle for an SH group which can readily become acylated to form an acyl thiol ester.

Now we come to the second function of the sparker. These thioesters —the fatty acyl derivatives of CoA—are oxidized by repetitions of a cyclical process which has been called the β-oxidation cycle (15) (Fig. 2). At each turn of the cycle, acetyl CoA is liberated. For reasons which will become clear later, acetyl CoA cannot accumulate as such without bringing the process to a quick stop. The sparker in the course of oxidation gives rise to oxalacetate which condenses with acetyl CoA to form citrate, CoA being liberated in the process. This condensation reaction, discovered and documented so brilliantly by Ochoa and his group (16), may be considered as a device not only for regenerating coenzyme A needed in the initial activation of fatty acids but also for perpetuating

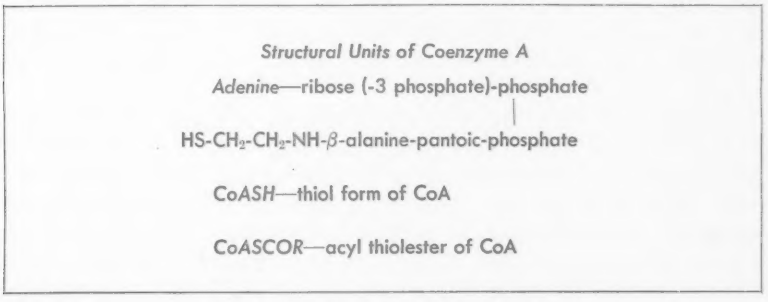

Fig. 1. Structural units of coenzyme A.

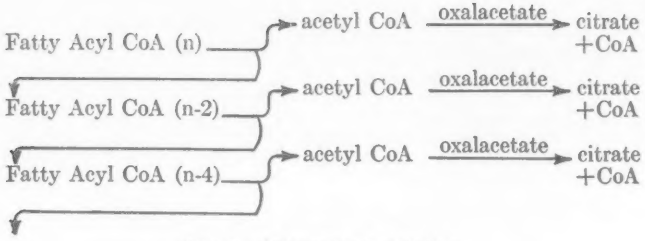

Fig. 2. Scheme for β-oxidation.

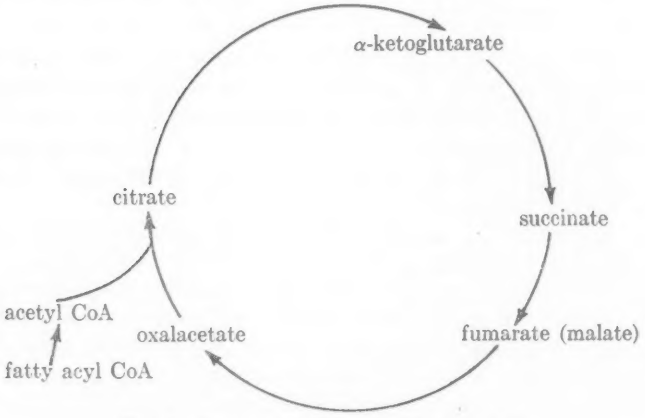

Fig. 3. Citric and fatty acid oxidation cycles.

sparker (Fig. 3). Citrate formed as the product of the condensation can now undergo oxidation back to oxalacetate through α-ketoglutarate, succinate, and malate as intermediates. This is in fact the pathway of the citric acid cycle. In addition each one of these intermediary oxidative steps generates ATP needed for the initial activation of the fatty acid. Thus the conversion of one mole of citrate to oxalacetate is attended by the formation of some 12 moles of ATP.

In theory, at any rate, a catalytic amount of sparker should spark the oxidation of an unlimited amount of fatty acid. Once initiated the process should be self-perpetuating. In practice that is not entirely true. Some fraction of the total number of molecules of oxalacetate formed undergoes decarboxylation to pyruvate or transamination with glutamate or other amino acids. There is, in other words, a constant loss of sparker in side reactions. Thus, to keep the fatty acid oxidation pot

always on the boil, a not inconsiderable amount of sparker must be present at all times.

Acetyl CoA cannot accumulate as the end product of fatty acid oxidation in mitochondria since CoA is present only in catalytic amounts. Thus, unless CoA can go through a cycle of esterification and deesterification, the oxidation would grind to a halt as soon as all the CoA became esterified. The citrate condensation step is one device for release of coenzyme A (16). We shall discuss later the formation of acetoacetate as another device evolved by liver for accomplishing the same purpose. But we will forego consideration of acetoacetate formation until the individual enzymes of fatty acid oxidation have been discussed in more detail.

THE STEPS OF FATTY ACID OXIDATION

It is essentially impossible to recognize the individual steps of fatty acid oxidation in the mitochondrial system. This system is designed for the initial fatty acids to go directly to CO_2 and water without accumulating more than catalytic amounts of intermediary products. Two preliminary steps were necessary for the study of intermediates and of the enzymes which accomplish these transformations: ways and means had to be found of releasing the individual enzymes of the fatty acid oxidation system from mitochondria, and at the same time, methods and technics had to be devised for studying one-step reactions. In point of fact until appropriate methods became available there was no way of recognizing the individual enzymes involved in one-step reactions.

Availability of Coenzyme A

Fatty acid oxidation as it proceeds in mitochondria involves catalytic amounts of CoA and an elaborate cycle to regenerate CoA. If one had to reproduce such an arrangement for regeneration of CoA it would be virtually impossible to study in a test tube any one enzyme process in simple fashion. Clearly the proper substrate of each of the enzymes has to be provided in excess, and that means preparing the various acyl derivatives of CoA in substrate amounts. There are two different ways of accomplishing this preparative task. The first involves the use of the very enzymes which carry out the task in the mitochondrion—in other words, the activation enzymes which carry out the ATP-catalyzed esterification of CoA by fatty acids or by fatty acid derivatives as shown

in Equation 1:

1. ATP + CoASH + fatty acid $\rightleftharpoons$ S-fatty acyl CoA + AMP + inorganic pyrophosphate (11, 12)

The second is that of chemical synthesis of which there are now three variations shown in Equations 2–4:

2. Fatty anhydride + CoASH $\rightarrow$ S-fatty acyl CoA + fatty acid (17)
3. Fatty acyl thiophenol + CoASH $\rightarrow$ S-fatty acyl CoA + thiophenol (18)
4. Fatty thioacid + CoASH $\rightarrow$ S-fatty acyl CoA + H_2S, (19)

One of the simplest involves the condensation of fatty anhydrides with SHCoA in bicarbonate solution. Other methods make use of thioacids or acylthiophenols as the acylating agents for coenzyme A.

I should add parenthetically that none of these preparative methods would be practical unless coenzyme A were available in relatively large amounts. It is perhaps no coincidence that the solution of the problem of reconstructing fatty acid oxidation followed almost immediately on the publication of a method for large-scale isolation of coenzyme A which made possible commercial production of this hitherto rare coenzyme on an almost unlimited scale (20).

Isolation of Mitochondria

When aqueous mitochondrial suspensions are treated with some 10 volumes of acetone the resulting acetone powder contains dried, damaged mitochondria which when extracted with dilute salt solutions readily release the enzymes of the fatty acid oxidation cycle (21, 22). These enzymes after solubilization can now be separated one from another by the conventional methods of protein purification.

Since the enzymes in question are localized in mitochondria it is obvious that great initial purification can be obtained by first separating particles generally from the soluble constituents of the cell and then separating mitochondria by differential centrifugation from other cell particulates. While the isolation of mitochondria by this type of procedure had been accomplished some years previously through the efforts of Hogeboom and Schneider (23) the technic had been applied exclusively to tissues of laboratory animals. In our own laboratory it has been found possible to prepare mitochondrial suspensions suitable for isolation of enzymes on a very large scale from slaughter-house material (11, 24). This development has simplified enormously the task of purifying the enzymes of fatty acid oxidation.

The Four Basic Reactions

Even Numbered Fatty Acids

Now let us make an over-all survey of the four basic reactions in the fatty acid oxidation cycle as summarized in Equations 5–8:

5. $RCH_2CH_2COS\overline{CoA} \xrightarrow{-2H} RCH{=}CHCOS\overline{CoA}$ (25, 27)
6. $RCH{=}CHCOS\overline{CoA} + H_2O \rightarrow RCHOHCH_2COS\overline{CoA}$ (28, 29)
7. $RCHOHCH_2COS\overline{CoA} \xrightarrow{-2H} RCOCH_2COS\overline{CoA}$ (15, 30)
8. $RCOCH_2COS\overline{CoA} + CoASH \rightarrow RCOS\overline{CoA} + CH_3COS\overline{CoA}$ (15, 31)

The fatty acyl CoA is oxidized by a flavoprotein enzyme to the corresponding α-β unsaturated acyl CoA derivative (25). In turn this unsaturated derivative is hydrated to form the L configurational β-hydroxyacyl CoA (32). Then a second oxidation takes place at the β-carbon atom leading to the formation of the β-ketoacyl CoA (33, 34). Finally the β-ketoacyl CoA is cleaved in the presence of a molecule of free CoA, resulting in two products of cleavage, acetyl CoA and a fatty acyl CoA with two carbon atoms less than the parent fatty acyl CoA (15, 31).

This new acyl CoA now undergoes a repeat of the four above mentioned reactions while acetyl CoA is condensed with oxalacetate to form citrate. These four reactions constitute one turn of the β-oxidation cycle which continues until eventually all the fatty acid molecule has been transformed into acetyl CoA (Fig. 4). This point is reached when the β-ketoacyl CoA has only four carbon atoms. The cleavage of this derivative leads to two molecules of acetyl CoA and no residue is left.

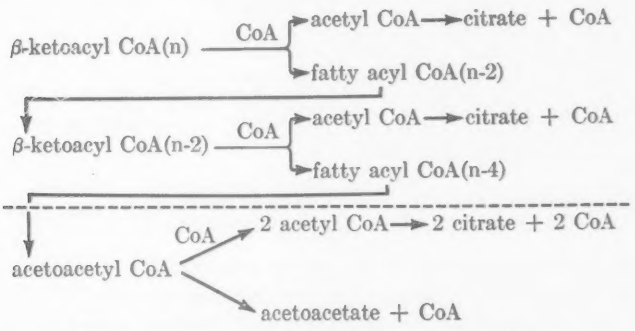

Fig. 4. Alternative pathways of fatty acid degradation.

Odd Numbered Fatty Acids

Let us consider what happens when a fatty acid with an odd number of carbon atoms is subjected to the β-oxidation sequence. Successive degradation eventually leads to a five carbon β-ketoacyl CoA which on cleavage yields acetyl CoA and propionyl CoA. There is no possibility of further reaction for propionyl CoA in the case of mitochondria other than from liver. Deacylation of propionyl CoA by enzymes present in kidney and heart mitochondria yields propionic acid which accumulates as such.

Action of Liver Mitochondria

In the case of liver mitochondria an alternative pathway is provided at the stage of acetoacetyl CoA (Fig. 4). In addition to the possibility of cleavage to two molecules of acetyl CoA, acetoacetyl CoA can be deacylated to form acetoacetate and free CoA (35). When any reagent which interferes with the citric acid cycle, such as malonate, is added to liver mitochondria (5, 6) or when a physiologic situation exists—as in diabetes mellitus—where generation of members of the citric acid cycle is interfered with or suppressed, then the deacylation mechanism becomes the exclusive mechanism for disposal of acetoacetyl CoA. The accumulation of the so-called acetone bodies (derived from acetoacetic acid) is a token that the level of sparker in liver has been reduced well below normal.

There is a very simple explanation for the fact that acetoacetate accumulates only in liver. Liver is by no means the only tissue which contains an enzyme capable of deacylating acetoacetyl CoA. However, liver is the only known tissue which lacks an enzyme capable of converting acetoacetate to acetoacetyl CoA in presence of ATP and CoA. Thus while other tissues can form acetoacetate, no accumulation of acetoacetate can be observed since it is pushed back into the metabolic hopper as fast as it is pushed out.

Thus far we have skirted around the edges of the individual enzymes of the β-oxidation cycle and I propose now to consider in more detail each of the pertinent enzymes.

THE KNOWN INDIVIDUAL ENZYMES

Acylation

There are four known enzymes which catalyze the acylation of CoA by fatty acids or by substituted fatty acids in presence of ATP (Table 3). One is specific for acetic and propionic acids (24, 35, 36); a second acts upon fatty acids from C_4 to C_{12} and upon a wide variety of substituted,

Table 3. Fatty Acid Activation Enzymes

Designation	*Chain-length range*	*Specificity*
Acetate (heart)	C_2, C_3	Fatty acids
Mahler-Wakil enzyme (liver)	C_4–C_{12}	Fatty acids + branched, phenyl, β-hydroxy, α-β
Kornberg-Pricer (liver)	C_{10}–C_{18}	Fatty acids + . . .
β-Ketoacid (kidney)	C_4–C_{12}	β-ketoacids

branched, and phenyl fatty acids (11); a third acts upon fatty acids in the range of chain length from C_{10} to C_{18} (12); while a fourth acts generally upon β-ketoacids—a species of substituted acid which is left severely alone by the other enzymes in the group (37). ATP is the only nucleotide which can initiate the acylation and even adenosine diphosphate (ADP) is inactive in this regard. The precise mechanism by which the acylation of CoA is initiated by ATP is still undetermined. In a general way it may be assumed that ATP reacts with the activating enzyme to form pyrophosphoryl enzyme with liberation of adenosine monophosphate (AMP). A series of replacements then take place: fatty acid for the pyrophosphoryl group, and then CoASH for the enzyme. The net reaction is then the conversion of ATP to AMP and P-P, coupled to the acylation of CoA by the fatty acid (cf. Equations 9–11):

9. ATP + enzyme → pyrophosphoryl enzyme + AMP
10. Pyrophosphoryl enzyme + fatty acid + fatty acyl enzyme + pyrophosphate
11. Fatty acyl enzyme + CoASH + S-fatty acyl CoA + enzyme

Jones *et al.* (38, 39) have proposed another mechanism in which the interaction of ATP with enzyme leads to the formation of adenosine-monophosphoryl enzyme with liberation of inorganic pyrophosphate.

The acylation reaction is readily followed by measuring the SH function of CoA. As acylation proceeds the SH group of CoA disappears. The delicate nitroprusside reaction for free SH groups serves as the basis of a very simple and convenient method of assay of the activation enzymes (11).

It should be mentioned that the activation reaction is a reversible one and that the equilibrium constant for the reaction at 30° is about 1 (11).

Dehydrogenation of Fatty Acyl CoA's

There are two known enzymes which catalyze the dehydrogenation of fatty acyl CoA's: a green copper-containing flavoprotein which acts

in the C_4 to C_8 chain length range (25, 40), and a yellow, iron-containing flavoprotein which acts on all acyl CoA's from C_4 to C_{18} (26, 35). It can readily be demonstrated that the flavoproteins are reduced coincident with the oxidation of the substrate. The metal plays no role in the primary oxidation of the substrate, as shown by the fact that preparation of these flavoproteins which have been freed of metal are still readily reducible by substrate. The metal is concerned with interaction of the reduced flavoprotein enzyme with one-electron acceptors (40, 41).

The interaction of the two flavoproteins with electron acceptors is a complex process which will be discussed in detail later on. For present purposes it is sufficient to stress that there are two important problems which remain unsolved with respect to the mode of action of the fatty acyl CoA dehydrogenases: first, which oxidant oxidizes their reduced forms physiologically, and second, which reductant reduces them in their oxidized form when the reaction is reversed as in synthesis of long-chain fatty acids from acetyl CoA. The enzymatic oxidation of the fatty acyl CoA's is a reversible process. The oxidation-reduction potential at *p*H 7 is about +0.2V which is the most positive E'_o known for a substrate system (40).

There is no one simple method of following the action of the fatty acyl CoA dehydrogenases which is free from complication or possible sources of error. To make matters worse the interaction of the primary dehydrogenase with electron acceptors is not a direct process and thus does not lend itself to a simple assay. But in a general way the direct reduction of the enzyme by the substrate is the least ambiguous way of demonstrating the dehydrogenase and this can be made the basis of a method for evaluating purity levels (26).

The unsaturated acyl CoA hydrase acts on α,β- and β,γ-unsaturated acyl CoA's regardless of chain length (28). The hydration reaction is reversible. At equilibrium three species are present—the α,β-unsaturated acyl CoA, the β,γ-counterpart, and the β-hydroxyacyl CoA.

The oxidation of L(+),β-hydroxyacyl CoA's by diphosphopyridine nucleotide (DPN) is catalyzed by an enzyme which acts upon derivatives covering the entire gamut of chain length according to Equation 12 (30):

12. β-Hydroxyacyl CoA + $DPN^+ \rightarrow$ β-ketoacyl CoA + DPNH + H^+

The reaction is readily followed kinetically by observing the change in absorption at 340 mμ which accompanies reduction of DPN (15, 30).

At neutral *p*H the equilibrium of the reaction lies almost completely to the left while above *p*H 9 the equilibrium is shifted almost completely to the right. This fact is taken advantage of as the basis of a method for preparing β-ketoacyl CoA's.

The estimation of hydrase is based on the coupling of the hydrase reaction with the subsequent dehydrogenation reaction at *p*H 9 (28). The rate of reduction of DPN can be used as a measure of the rate of hydration of the unsaturated acyl CoA providing the β-hydroxyacyl CoA dehydrogenase is present in excess.

The β-ketoacyl CoA cleavage enzyme acts upon derivatives from C_4 to at least C_{12} (31). The reaction probably proceeds through the sequence shown in Equations 13–14 below:

13. $RCOCH_2COSCoA + Enzyme \rightarrow RCO\ Enzyme + CH_3COSCoA$,
14. $RCO\ Enzyme + CoASH \rightarrow RCOSCoA + Enzyme$.

The enzyme breaks the carbon-to-carbon bond between the α and β carbon atoms of the β-ketoacyl CoA—the acyl group becoming attached to the enzyme while the two-carbon fragment is liberated as acetyl CoA. The acyl enzyme complex is then dissociated by coenzyme A with formation of acyl CoA and liberation of the free enzyme. This process has been aptly called thiolysis by Lynen (33).

The cleavage of acetoacetyl CoA to two molecules of acetyl CoA has been shown to be reversible. The equilibrium point lies almost completely to the side of acetyl CoA (42).

Reductive Synthesis of Fatty Acids

It is of great importance to know whether physiologically the same group of enzymes which catalyze the oxidative breakdown of fatty acids can also bring about the reductive synthesis of long-chain acids from acetyl CoA. Since all the reactions of the fatty acid cycle have been shown to be reversible it should in principle be possible to accomplish synthesis by reversal of the reactions of the β-oxidation cycle.

A partial synthesis has already been reported by Beinert and Stansly (43) who were able to demonstrate the formation of butyryl CoA from acetyl CoA in presence of the four enzymes of the cycle under conditions suitable for synthesis. The key to synthesis lies in the maintenance of reducing conditions. The two oxidative steps can be reversed only when appropriate reducing agents are present.

The problem of the second oxidative step is a simple one since the physiologic electron acceptor is known to be DPN^+. To reverse this

oxidation step it is merely sufficient to introduce a dehydrogenase system which can generate DPNH and there are many systems known which can serve in that capacity.

The reversal of the first oxidative step is a somewhat more sticky problem. Cytochrome *c* is the only natural acceptor which is reducible by the acyl CoA dehydrogenase. While there are dehydrogenase systems available which can maintain cytochrome *c* in the reduced state it has been assumed on the basis of oxidation-reduction potential consideration that the head of potential between reduced cytochrome *c* and the acyl CoA dehydrogenase system would not be sufficient to drive the reduction efficiently. In consequence Beinert and Stansly used reduced benzyl viologen as the driving force for the reduction of the unsaturated acyl CoA to the fatty acyl CoA. Benzyl viologen is an artificial acceptor of very negative oxidation-reduction potential which is readily reduced by any of several dehydrogenase systems such as milk xanthine oxidase.

The fact that butyryl CoA was synthesized from acetyl CoA in the Beinert-Stansly system rules out the possibility of inadequate reducing conditions. The failure to synthesize fatty acyl CoA's higher than butyryl CoA must be ascribed to another cause. There is reason to believe that the fault lies with the cleavage enzyme. The equations describing the synthesis of β-ketoacyl CoA's by reversal of the cleavage reaction are shown in Equations 15–17 below:

15. Acetyl CoA + acetyl CoA → β-ketobutyryl CoA + CoA
16. Acetyl CoA + butyryl CoA → β-ketocaproyl CoA + CoA
17. Acetyl CoA + fatty $acyl_nCoA$ → β-$ketoacyl_{n+2}CoA$ + CoA

If the cleavage enzyme is presented with a mixture of acetyl CoA and butyryl CoA three possibilities of reaction exist. Either Reaction 15 or 16 or both can take place. The particular cleavage enzyme which Beinert and Stansly worked with carried on only Reaction 15 when presented with a mixture of acetyl CoA and butyryl CoA. The explanation for this selectivity is probably as follows. The cleavage enzyme carries out a condensation between acetyl CoA and a fatty acyl CoA. If the affinity of the cleavage enzyme for the fatty acyl CoA increases sharply with decreasing chain length then the condensation of acetyl CoA with the shortest fatty acyl CoA—another molecule of acetyl CoA—will take complete precedence over the condensation of acetyl CoA with a higher fatty acyl CoA such as butyryl CoA.

Popjak and Tietz in England (44) and Gurin *et al.* (45, 46) in this country have been able to show synthesis of long-chain fatty acids from acetate in homogenates and extracts of mammary tissue and liver respectively. It is possible that there are two types of cleavage enzyme.

The first type which is important in oxidation has the characteristics noted by Beinert and Stansly. The second which is important in reductive synthesis may show highest affinity for the long-chain fatty acyl CoA's. That would mean that such a cleavage enzyme would preferentially form long-chain β-ketoacyl CoA's rather than acetoacetyl CoA.

ISOLATION OF DEHYDROGENASES OF ACYL CoA's

The two enzymes which catalyze the dehydrogenation of acyl CoA's to their unsaturated derivatives can be isolated in the form of a complex in which these two flavoproteins are linked to still another flavoprotein which Beinert and Crane have called the electron-transferring flavoprotein (47). The functional interrelationships of these three flavoproteins may be represented diagrammatically, as in Fig. 5. The interaction of the two dehydrogenases with electron acceptors is not direct but has to go through the electron-transferring flavoprotein as an intermediary. Electrophoresis is essentially the only effective procedure for separating the two dehydrogenases from one another and from the electron-transferring flavoprotein. However, the three enzymes form a very tightly associated complex which is not resolved by any of a large number of preparative procedures.

First, let us consider the significance of there being two separate enzymes in the same complex with the same catalytic function which differ only in the range of substrate chain length over which they are effective. The yellow dehydrogenase is active on acyl CoA's from C_4 to C_{18}, but the affinity of the enzyme for the substrate increases with increasing chain length (26). The properties of the yellow dehydrogenase are such that when presented with a mixture of acyl CoA's it will prefer to oxidize the derivatives of longer chain length at the expense of the shorter chain derivatives. This would lead to an undesirable situation under physiologic conditions, namely the accumulation of shorter-chain acyl CoA's and the consequent tying up in enforced idleness of valuable coenzyme A. The green dehydrogenase solves this particular dilemma. At the point in chain length where a fatty acyl CoA cannot compete favorably with the C_{18} derivative (about C_8), it can be acted upon by the green dehydrogenase without interference by the long-chain acyl CoA's since the affinity of the green dehydrogenase for acyl CoA's decreases as the chain length of the acyl CoA's increases.

It is entirely possible that the device of a complex of two enzymes with similar catalytic activities but with different affinities with respect to substrate chain length is not confined to the acyl CoA dehydrogenases but may have its counterpart in all the other enzymes of the β-oxidation sequence. This possibility has yet to be tested experimentally.

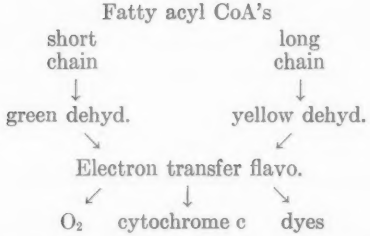

Fig. 5. Electron transfer scheme. According to H. Beinert and F. Crane.

Finally may I call attention to the fact that in the complex of the three flavoproteins which are concerned with the oxidation of fatty acyl CoA's we have a model in miniature of the kind of forces which hold the mitochondrion together and of the remarkable way in which juxtaposition of enzymes with serial function minimizes the random diffusion of substrate molecule from one enzyme to another.

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American Association of Clinical Chemists

President's Address

Monroe E. Freeman

OVER THE PAST FEW YEARS The American Association of Clinical Chemists has established an outstanding record of progress and achievement. Today, the state of the Association is excellent. For the future, there are ambitious plans and favorable prospects. The past history, the objectives, and accomplishments of the Association are familiar to the readers of the CLINICAL CHEMIST and detailed review would add little. However, it is interesting to note, in passing, that Dr. Max M. Friedman's address[1] to the Association in 1949 forecast the many important tasks to be done and the subsequent record of accomplishment is convincing evidence of the rapidly growing strength of the Association.

Rather than detail the record of accomplishment, it seemed more appropriate to examine the fundamental elements of purpose that seem to have contributed the most to the success of this enterprise. It is these simple fundamentals that should be the soundest guidance for future efforts.

First, there should be a real need for an organization of clinical chemistry. Second, there must be a comprehensive program to fill that need. Third, there must be individuals who are willing and able to effectively implement the program.

At first glance, it might seem unnecessary to debate the need for such an organization in the field of modern medical science. Certainly the members of this association as well as a large segment of the medical profession would not dissent, but at the same time there has been a body of contrary opinion. Opinion, pro or con, however, counts for little in the face of such facts as the rapid growth of the American Association, the

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Lt. Col., Medical Service Corps.

Delivered at the Annual Meeting, New York, September 17, 1954.

[1] *Chem. & Eng. News* **27**, 3116 (1949).

rise of similar organizations throughout Western Europe, recognition by other scientific societies, and the assembly of 300 clinical chemists from 23 nations and five continents at the First European Congress of Clinical Chemistry.

From this positive assertion of the need for an organization of clinical chemistry, we can derive two specifications. First, in the public interest, there is a need for an organized effort to advance the science and practice. It is obvious that the increasing complexity of chemical theory and technic involved in modern medical practice requires a comprehensive organization of the pertinent and applicable chemical knowledge. Second, in the interests of the individual chemist, there is a need for a strong organization to protect and improve his professional identity, and to facilitate his professional improvement through educational guidance and the free exchange of technical information. This requirement is particularly significant in the newly developing field of clinical chemistry because the individual should not carelessly nor inadvertently trespass in the field of medical practice and medical responsibility, nor should he retreat into the circumscribed area of mere technical analysis. Authoritative guidance is needed to help the clinical chemist clearly define his position in the field of medical science.

The second element of purpose was a comprehensive program, and a program has been derived from Article II of the Constitution. Since few people read Constitutions, quotation will not be superfluous:

> In the interest of the public welfare, it is the aim and object of this association to raise the level at which chemistry is practiced in the clinical laboratories to stimulate the development of new methods for use in the clinical laboratories; to encourage highly trained chemists to enter the field of clinical chemistry; to encourage those engaged in this field to pursue advanced studies so as to more effectively render service to the public; and to create and maintain a forum where chemists engaged in applying the science of clinical chemistry may exchange ideas and information concerning their scientific, technical, and professional problems.

These are fine words but they mean little unless effectively implemented by positive action. In short, what have we done? What are we doing? and What do we intend? This deserves special emphasis because there is some reason to believe that scientific societies may be approaching a "buyer's market." There are many worthwhile scientific organizations, so many that few young scientists can afford membership in all. They will become more critical in their selections and it is only logical to assume that the best of the younger generation will be attracted by real accomplishment and by prospects of progressive action.

Illustrations of accomplishment and current activities include; the first volume of *Standard Methods*, substantial progress on volumes two and three, and the new CLINICAL CHEMISTRY, important steps for a society as young as ours. Another most important accomplishment was the formalization of a Code of Ethics. The latter is a most significant step in defining the position of the clinical chemist, a position that has long been confused by controversy, prejudice, and legal restrictions that were, in themselves, capable of compounding confusion in forty-eight different ways. As the lines become more finely drawn in the next few years, it is most important that this Code of Ethics be maintained in a form that is acceptable and applicable to new conditions and situations. This is as much the responsibility of individuals and of the local sections as it is of the national officers and committees. It is essential that local sections maintain continued interest and surveillance over the Code of Ethics and critically review and recommend changes and improvements necessary or advisable.

By the same token, local sections and individuals must assume primary responsibility for the legislative problems that appear in their localities. The Association does not have the resources to support a central secretariat that could monitor the legislative proposals throughout the country. However, the national legislation committee under Dr. John G. Reinhold will provide competent advice and experienced counsel upon request. This committee will be most effective if it is kept fully informed regarding legislative matters in your locality so that the position and actions of the Association may be properly coordinated.

Other activities that are beginning to assume more significance are the employment exchange services and the personnel and laboratory standards. Some thought has been given to the problem of technical surveys. Standard samples have been circulated by different groups for different purposes, some for the evaluation of technician or laboratory proficiency, some for the evaluation of methods and primary standards. Surveys of this sort have been conducted by public health laboratories, by local sections of the association, by large laboratory systems such as the Army, and on an international scale under the sponsorship of the International Federation of Clinical Chemists. For the most part these have been independent and uncoordinated operations that could conceivably lead to as many different primary definitions and standards. Sooner or later, these efforts should be coordinated and reduced to a national or international definition of standards for materials, methods, and performance. This will be large task and a difficult one but there is no doubt that this

Association should be prepared to assume an appropriate share of the responsibility for such an effort.

The third element of success was the willingness of capable individuals to carry out the program. Special tribute must be paid to the leaders and officers of the past few years, to the committee members and to the individuals who have done the most in forwarding this enterprise. Most of them have done more than their share and continued responsibility would be an imposition. Their successors should represent the nation-wide scope of this Association, and their selection will result almost automatically from the interest and activity of the local sections in carrying on the program that has been so successfully begun.

Nominations for the 1955 Ernst Bischoff Award

The National Executive Committee of the American Association of Clinical Chemists has announced that the Association Award Committee will consider nominations for the 1955 Ernst Bischoff Award in Clinical Chemistry.

The Ernst Bischoff Award is presented annually to an outstanding clinical chemist selected by the Association. The Award consists of a scroll, a bronze medal, and an honorarium of $500.00. It was first presented in 1952, to Jos Kahn (posthumous). The other recipients have been Michael Somogyi (1953) and John G. Reinhold (1954).

Candidates for this award are proposed by the membership of the A.A.C.C. A member may place in nomination the name of any individual by submitting in triplicate a letter of recommendation and any other pertinent data relative to the nomination to the Chairman of the Award Committee. Nominations must be received on or before March 30, 1955, to be considered for the 1955 Award.

Nominations should be sent to Dr. Joseph H. Roe, 1335 H. St., N. W., Washington, D. C.

Executive Committee Meeting

The National Executive Committee of the American Association of Clinical Chemists met at the Mount Sinai Hospital, New York, on September 15, 1954. Present were Monroe E. Freeman, *President*; Max M. Friedman, *National Secretary*; Louis B. Dotti, *National Treasurer*; Robert M. Hill, Hugh J. McDonald, Marschelle H. Power, Miriam Reiner, Albert B. Sample, Harold D. Appleton (by invitation), John G. Reinhold (by invitation), Joseph I. Routh (by invitation), and Harry Sobotka (by invitation).

The Treasurer's report was given for the fiscal year July 1, 1953–June 30, 1954.

Income	
Membership dues	$3736.30
Clinical Chemist	1136.25
Membership list	50.00
Certificates	20.00
Bank interest	32.48
TOTAL INCOME	$4975.03
Expenses	
Clinical Chemist	$3754.22
Treasurer	88.41
Secretary	321.24
Executive Committee	100.40
Membership Committee	56.00
Section allotments	50.10
Membership fee refunds	8.20
TOTAL EXPENSES	$4378.57
Balance for year	$596.46
Bank balance, as of June 30, 1953	1911.67
Unaccounted credit	10.00
Bank balance July 1, 1953	$2518.13

John G. Reinhold reported for the Nominating Committee and recom-

mended the following slate of officers for 1955–56:

President: Otto Schales

Vice-President: Robert M. Hill

National Secretary: Max M. Friedman

National Treasurer: Louis B. Dotti

Executive Committee: Monroe E. Freeman, Joseph H. Gast, William H. Goldwater, Icie M. Hoobler, and Samuel Natelson.

REPORTS AND ACTIVITIES

The Committee on Membership reported a total of 624 members in the Association as of September 15, 1954.

Nelson F. Young reported for the editorial board working on Volume II of *Standard Methods* that the manuscript for this volume is nearly complete. Kurt M. Dubowski reported that work has already been started on Volume III of *Standard Methods* and will include toxicologic technics for use in hospital laboratories as emergency procedures.

Albert E. Sobel reported that a collaborative program has been started with the American Association for the Advancement of Science and approval has been received for a symposium as well as a series of contributed papers to be presented at the Atlanta meetings on December 26–31, 1955.

Oliver H. Gaebler reported that the Intersociety Committee met in Atlantic City on April 11, 1954, and has tentatively scheduled the next meeting for Buffalo, N. Y.

Otto Schales reported that John G. Reinhold was voted the recipient of the third Ernst Bischoff Award for his outstanding contributions to clinical chemistry.

Max M. Friedman reported that the committee appointed to recommend revision of the constitution is now progressing in its study.

Joseph I. Routh reported that the activity of the Employment Clearing House had increased considerably and various openings for clinical chemists are now being handled.

Emmett B. Carmichael reported that the Committee on Education was now organized and a program will soon be under way.

Robert L. Dryer reported for the Committee on Laboratory Standards and Personnel. A questionnaire had been distributed and the returns are now being evaluated.

NEW COMMITTEES

The following committees were appointed: *Program Committee:* Marschelle H. Power, chairman; *Ernst Bischoff Award:* Joseph H. Roe, chairman; Ferrin B. Moreland and Arnold G. Ware; *Committee on Ethics:* Hugh J. McDonald, chairman; Albert L. Chaney and Harry Sobotka. *Legislative Committee:* John G. Reinhold, chairman.

George T. Lewis was invited to join the Board of Editors of CLINICAL CHEMISTRY.

RESOLUTIONS PASSED

It was moved, seconded, and passed that only five issues of *The Clinical Chemist* shall be published during 1954 so as to save expenses and conserve

scientific material for the new journal, CLINICAL CHEMISTRY.

It was moved, seconded, and passed that the bylaws of the Chicago, Midwest and Washington-Baltimore-Richmond sections be approved, with the recommendation to the sections that their local officers be designated as chairman and vice-chairman rather than president and vice-president, respectively, in order to avoid confusion between national and local offices.

It was moved, seconded and passed that membership shall be terminated for those who have not paid their annual dues by March 15 of any calendar year and their subscriptions to CLINICAL CHEMISTRY withdrawn.

It was unanimously agreed that an invitation from the American Society of Biological Chemists for a joint symposium at the Federation meetings shall be accepted.

The Executive Committee agreed to propose to the membership that the dues for 1955 include the assessment for the journal.

It was moved, seconded, and passed that the seventh annual meetings of the A.A.C.C. shall be held in conjunction with the American Chemical Society in Minneapolis, Minnesota, from September 11–16, 1955.

ABSTRACTS

Editor: Ellenmae Viergiver. *Contributors:* Joseph A. Annino, Clyde A. Dubbs, Alex Kaplan, Margaret M. Kaser, Miriam Reiner, Herbert Thompson

Studies of the thymol turbidity, cephalin flocculation, and thymol flocculation tests. E. J. Katz, R. J. Hasterlik, and F. E. Snapp (*Health Services Division and the Division of Biological and Medical Research, Argonne National Laboratory, Lamont, Ill.*)

Four thymol reagents were studied: (I) Maclagen reagent, *p*H 7.72; (II) thymol reagent modified by Huerga and Popper, *p*H 7.72; (III) Maclagen reagent modified by Mateer *et al.*, *p*H 7.55; and (IV) thymol reagent modified by Huerga and Popper, *p*H 7.55.

Calculation of thymol turbidity measurements was based on the precalibrated colloidal glass standard of Reinhold. Results indicated that Reagent II was best for screening both normal individuals and patients with liver disease. The thymol flocculation test, regardless of the reagent used, was unsatisfactory as a screening test. Two cephalin cholesterol antigens, Difco and Wilson, were compared, and it was found that the latter gave fewer positive results in normal individuals and patients ill with diseases other than liver disease. Results in patients with liver disease were approximately the same with both antigens.—*J. Lab. & Clin. Med.* **44,** 353 (1954).

E.V

Clinical value of the plasma butanol-extractable (thyroxin) I^{131} in the diagnosis of hyperthyroidism and myxedema. A. L. Schultz, S. Sandhaus, H. L. Demorest, and L. Zieve (*Veterans Admin. Hosp., Minneapolis, Minn.*)

Butanol-extractable I^{131}: A 5 ml. aliquot of plasma is extracted twice with 20 ml. of *n*-butanol and three times with one half volume of 4N NaOH which contains 5 per cent Na_2CO_3 to remove the nonthyroxine I^{131} carried into the butanol phase. The butanol extract is concentrated over a waterbath and adjusted to a volume of 6 ml. Radioactivity is counted in a well-type scintillation counter.

Plasma protein-bound I^{131}: A 5 ml. aliquot of plasma is placed in a flat-bottom 20 ml. counting tube. Proteins are precipitated with 10 ml. of 20 per cent trichloroacetic acid. After centrifugation, the proteins are washed four times with trichloroacetic acid, care being taken not to pour off or remove protein particles. The solidly packed protein is then counted in a well-type scintillation counter.

Butanol-extractable I^{131} and protein-bound I^{131} levels in plasma 24 hours after administration of a tracer dose of I^{131} were studied in 50 euthyroid, 12 hyperthyroid, and 5 myxedematous subjects. The two measures are closely correlated, the butanol-extractable fraction comprising four fifths of the protein-bound

iodine, but has no diagnostic significance. Euthyroid individuals can be separated from hyperthyroid cases, but the test is not useful in distinguishing myxedematous patients.—*J. Clin. Endocrinol. & Metab.* **14,** 1062 (1954).

M. R.

Comparison of incineration and chloric acid methods for determination of chemical protein-bound iodine. L. Zieve, M. Dahle, and A. L. Schultz (*Dept. of Medicine, and the Radioisotope Unit, Veterans Administration Hospital and the Univ. of Minnesota, Minneapolis, Minn.*)

A comparison was made between the values obtained with an incineration method and a chloric acid method. Although there was no significant difference between the two methods in hypothyroid and hyperthyroid groups, it was found that euthyroid values obtained by the chloric acid method were shifted upward approximately 1–1.5 μg. per 100 ml. Since the hyperthyroid levels were not shifted correspondingly, there was a loss in discriminative effectiveness of the test. The chloric acid procedure did not prove to be significantly more rapid to perform in terms of actual technician-time spent on the procedures.—*J. Lab. & Clin. Med.* **44,** 374 (1954).

E.V.

An improved method for multiple rapid determinations of arterial blood *p*H. D. A. Holaday (*Dept. of Anesthesiology, Columbia University, College of Physicians & Surgeons, and the Anesthesiology Service, Presbyterian Hospital, New York*)

Details of construction are presented for an instrument which permits direct transfer of blood from the patient into a glass electrode for serial determinations of *p*H at body temperature. The volume of blood required for each determination is approximately 2 ml. The apparatus is designed for use in the presence of explosive gas mixtures in the operating room.—*J. Lab. & Clin. Med.* **44,** 149 (1954).

A.K.

Conductometric titration method for determination of serum chloride. H. A. Teloh (*Evanston Hospital Association, Evanston, Ill., and Northwestern University Medical School, Chicago, Ill.*).

Proteins are precipitated from 0.5 ml. of serum by the addition in drops of 5 ml. of absolute alcohol. After vigorous shaking, the tube is centrifuged at 1500–2000 r.p.m. for 2 minutes. The supernatant liquid is decanted into a 45 ml. Pyrex glass-stoppered Freas conductance cell. Five milliliters of absolute alcohol is added to the precipitate and the contents are thoroughly mixed by shaking and stirring. After centrifuging, the supernatant is also added to the Freas cell, followed by approximately 40 ml. of double-distilled chloride-free water. The contents of the cell are mixed by shaking and brought to room temperature. The cell is connected to the conductance measuring apparatus (H. G. Davis, *to be published*) and the conductance measured after consecutive additions of

0.1 ml. of 100 mEq./l. $AgNO_3$ solution. Contents of the cell should be mixed after each addition. The end point, graphically calculated, is associated with an abrupt and marked increase in conductance. Sulfates, monohydrogen phosphates, and bicarbonates in concentrations up to 20–30 mEq./l. exerted no effect on the endpoint. Normal serum chloride levels by this method range from 97.4 to 106 mEq./l., with a mean of 101.0 mEq./l. Accuracy of this procedure was established in control experiments. Advantages of this method are not stated.—*Am. J. Clin. Pathol.* **24,** 1095 (1954).

H.E.T.

The control of variable influences on the electrolyte content of sweat and their application to clinical study of adrenal function. C. R. Kleeman, M. Quinn, and D. E. Bass (*Yale Univ. Med. School, New Haven, Conn.*)

Nonhormonal or local factors such as skin and air temperature, method of collection, rate and duration of sweating, and interval between the onset of sweating and its collection, play a fundamental role in determining the electrolyte composition of thermal sweat, irrespective of the level of adrenocortical activity. There is no reciprocal relationship between the concentrations of sodium (or chloride) and potassium in thermal sweat collected under controlled conditions from young, healthy males. When the nonhormonal or local influences on sweat are controlled, electrolyte concentrations in random samples of thermal sweat may serve as indices of adrenocortical activity.—*J. Clin. Endocrinol. & Metab.* **14,** 1126 (1954).

M. R.

Simultaneous determination of sucrose and inulin in biologic fluids. M. K. Young and J. F. Prudden (*Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Tex.*)

An anthrone procedure is presented for the determination of sucrose and the simultaneous determination of sucrose and inulin. The latter is accomplished by the measurement of the optical densities of samples before and after inversion and subsequent digestion with sodium hydroxide.—*J. Lab. & Clin. Med.* **44,** 160 (1954).

E.V.

Simplified sensitive test for thyroid function, using protein-bound I^{131}. L. Van Middlesworth, C. E. Nurnberger, and A. Lipscomb. (*Univ. Tennessee, Knoxville*)

Twenty-four hours after a 50 μc tracer dose of I^{131} is given, 5–10 ml. of venous blood is obtained. A 3–5 ml. plasma sample is placed in the 3 mm. annulus of a special plastic cup and the radiation is counted. Four to five drops of 3 per cent NaI carrier are delivered into the plasma, and 8–12 ml. of 20 per cent trichloroacetic acid is added. After centrifugation and washing twice with 10 ml. of 10 per cent trichloroacetic acid, the precipitate remaining in the plastic cup is counted. The conversion ratio equals $\frac{\text{count/min. of ppt.}}{\text{count/min. of plasma}}$ times 100. Conver-

sion ratios of over 50 per cent are considered indicative of hyperthyroidism, ratios of normal subjects are less than 50 per cent, ranging from 10 per cent to 30 per cent.—*J. Clin. Endocrinol. & Metab.* **14,** 1056 (1954).

M. R.

The use of substances depressing antithrombin activity in the assay of prothrombin. P. Fantl. (*Alfred Hospital, Melbourne, Australia*).

For the assay of prothrombin, a reaction mixture was prepared which consisted of 0.5 ml. of thromboplastin preparation (a 6% suspension of acetone-dried human or rabbit brain powder); 0.6 ml. of 0.025M $CaCl_2$; 0.45 ml. of 0.15M NaCl and 0.3 ml. of 0.18M pyrocatechol in Veronal buffer, *p*H 7.4. To this mixture was added 0.3 ml. of the material to be tested. At periods of 1 minute after the last addition, 0.2 ml. was blown into a mixture of 0.2 ml. of prothrombin-free human plasma (oxalated plasma stirred with 10–20 mg. $BaSO_4$ or $BaCO_3$ per ml. at 0° for 30 min.) and 0.05 ml. of 0.18M pyrocatechol and the clotting time determined. Reactions were carried out at 28° ± 1°. Values of 470–720 prothrombin units per ml. were found in an examination of 126 normal subjects.—*Biochem. J.* **57,** 416 (1954).

A.K.

The interaction of dyes with proteins on paper with special reference to paper electrophoresis. G. T. Franglen and N. H. Martin (*St. George's Medical School, London*)

The spectral absorption characteristics of bromophenol blue, bromocresol green, azocarmine B, and naphthalein black 12B have been examined. Azocarmine B is heterogeneous in chromatography on filter paper. Bromophenol blue slowly fades in 0.01N NaOH. The dye-binding characteristics of human albumin and human gamma globulin with bromocresol green have been studied individually and in mixtures of known composition. The results suggest that existing quantitative methods of analysis by paper-strip electrophoresis require reassessment. —*Biochem. J.* **57,** 626 (1954).

A.K.

Influence of hyperglycemic factor (Glucagon) on 17-hydroxysteroids in plasma and urine of man. P. P. Van Arsdel, Jr., H. H. Romizawa, and R. H. Williams (*University of Washington School of Medicine and King County Hospital, Seattle, Wash.*).

The subjects were 8 fasting hospital patients with normal renal and hepatic function and no signs of congestive heart failure. Four were given purified hyperglycemic factor in a dose of 10 μg./kg. body weight by intravenous infusion during a 30-minute period. Blood glucose and plasma 17-hydroxysteroids in samples taken just before and at the end of the infusion, and plasma steroids 1 hour after the start of the infusion, were determined. A 3-hour control urine specimen was collected prior to the infusion and a 2-hour specimen was collected during and after administration of the hyperglycemic factor. Seventeen-hydroxysteroids in

plasma were estimated by the method of Nelson and Samuels [*J. Clin. Endocrinol. and Metabolism* **12,** 519 (1952)] and in urine by the method of Glenn and Nelson [*J. Clin. Endocrinol. and Metabolism* **13,** 911 (1953)].

In the remaining 4 patients similar doses of hyperglycemic factor were injected intravenously during a 30-second period. Blood sugar was determined and the plasma hydroxysteroids were estimated before, at 20 minutes, and at 40 minutes after injection, and, in 2 patients, 80 minutes after injection. Urinary steroids were determined in 3-hour specimens. No significant changes in plasma steroid values occurred, and the results for urinary steroids were variable. No direct action of hyperglycemic factor on the adrenal cortex was demonstrated by these methods.—*Proc. Soc. Exp. Biol. Med.* **86,** 844 (1954).

M.K.

Diminished plasma 17-ketosteroid concentration in pregnancy. L. I. Gardner, R. L. Walton, W. Ellis, and E. C. Hughes (*State University of New York, Syracuse, N. Y.*).

Neutral 17-ketosteroids in plasma were determined by a method previously reported [Gardner, *J. Clin. Endocrinol. and Metabolism* **13,** 941 (1953)]. A control group of 17 men and 16 women showed a mean value of 61 μg. per 100 ml. of plasma with no significant sex difference. The mean for 30 pregnant women was 18 μg. per 100 ml., which was significantly lower than the controls ($p < .001$).—*Proc. Soc. Exp. Biol. Med.* **86,** 804 (1954).

M.K.

The light sensitivity of the Liebermann-Burchard reaction during spectrophotometric determination of cholesterol. J. K. Kabara (*Argonne Research Hospital, University of Chicago, Chicago, Ill.*)

The whole absorption curve of the color developed in the dark and in the light was investigated using cholesterol standards in a polar solvent (glacial acetic acid) and in a nonpolar solvent (toluene). The optical density of the two solutions, measured from 580 to 740 mμ, was the same in either solvent. The difference in shade that is seen is explained by the light sensitivity of the solution in the region of 450 mμ. Unless the range 340–450 mμ is used for the determination, no special precautions concerning light are necessary.—*J. Lab. & Clin. Med.* **44,** 246 (1954).

E.V.

Flame photometric determination of electrolytes in tissue and of calcium in serum. J. R. Denson (*Biochemistry Dept., Army Medical Research Laboratory, Fort Knox, Ky.*)

Flame photometric methods are presented for the simultaneous determination of calcium, magnesium, sodium, and potassium in tissue, and for the determination of calcium in blood serum. The instrument used was the Beckman Model DU with No. 9200 flame attachment.—*J. Biol. Chem.* **209,** 233 (1954).

E.V.

BOOK REVIEWS

Clinical Chemistry in Practical Medicine (ed. 4)

C. P. STEWART and D. M. DUNLOP. Baltimore, Md., The Williams and Wilkins Company, 1954, 320 pp., $5.00

The authors have set as their goal a discussion of the application of clinical chemistry to the everyday practice of clinical medicine. The book is intended to give the clinician and the senior medical student the knowledge of biochemical backgrounds where a chemical examination may be helpful, and the interpretation and significance to be placed on the laboratory results.

The reviewer has been impressed with the authors' awareness of the problems of the laboratory in their everyday dealings with the practicing physician. Throughout the book the reader is reminded of this; for example: "[though] chemical methods have established their right to be considered among the major aids of diagnostic medicine . . . [laboratory tests] supplement, but do not replace examination of the patient"; and ". . . if analysts are relieved of the necessity of doing useless work, the quality and therefore the value of the remainder is more likely to be maintained at a high level."

The chapters are well organized and the discussions are clearly presented. It must be remembered that the authors have always kept in mind their intended audience, and therefore the practicing biochemist and research worker may find the subject-matter presentation shallow.

Good discussions are found in the chapters "Water and Electrolyte Metabolism," "Neutrality Regulation," "Carbohydrate Metabolism," "Renal Function," "Plasma Proteins," "Tests of Gastric, Pancreatic and Hepatic Functions," "Cerebrospinal Fluid," and "Calcium and Phosphorus."

In the chapter "Thyroid Function," the basal metabolism determination is given preference. Under this heading there is also an adequate presentation of the use of radioactive iodine. The protein bound iodine determination is not mentioned.

The appendices contain a selection of simple methods which the practicing physician can perform as part of the office routine and a description of the clinical chemical procedures and laboratory methodology which are in use in the authors' laboratories.

Though references for further reading are not given, this book would make a good basic text for instruction of residents, interns, and technicians assigned to the clinical chemistry laboratory.

Chemotherapy of Infections

H. O. J. COLLIER. New York, John Wiley and Sons, Inc., 1954, 248 pp., $4.00

This small volume describes the general aspects of chemotherapy and the various groups of chemotherapeutic agents. Dr. Collier discusses the application of chemical substances used against microbes infecting the body, and though he admits that his book ignores many interesting substances, the chemical agents which are included are those in which important results have recently been achieved, and those compounds and experiments which clearly show the basic principles of chemotherapy and the historic development of these principles.

Dr. Collier adequately illustrates the newer approach to therapy which may be called "chemical pharmacology." The subject matter makes very interesting reading and does not demand a great background in either chemistry or microbiology. This book will prove interesting and instructive to those who have the authority to prescribe these drugs as well as those who wish to keep informed on the progress of a neighboring science.

A Practical Manual of Medical and Biological Staining Techniques

E. GURR. New York, Interscience Publishers, Inc., 1954, 320 pp. $4.00

This is an excellent manual dealing with preparation of material and staining procedures in all phases of microbiology. It is well written, giving the maximum information and directions in concise terminology.

The appendix contains formulas for stain preparation, tables of stain solubilities, and tables of refractive indices of the solvents and oils used in microbiological work.

This book is highly recommended as the laboratory supplement to the standard texts on microbiology and cytology.

Statistical Analysis in Chemistry and the Chemical Industry

C. A. BENNETT and N. L. FRANKLIN. New York, John Wiley and Sons, Inc., 1954, 724 pp. $8.00

This book has been sponsored by the Committee on Applied Mathematical Statistics, National Research Council of the National Academy of Sciences. The authors present those aspects of applied mathematical statistics which will be continually useful to both the chemist and chemical engineer. The subject matter is developed very completely with the theoretical basis, derivation of the methods, and the computational procedures. The reader is given a good background of mathematical theory so that he has a firm basis for judging application. The selection of methods is aimed specifically at the chemist and the examples given are taken from many branches of chemistry and chemical industry. The more recent developments in applied mathematical statistics have been included, with illustrations of their application to chemistry.

Human Biochemistry (ed. 4)

I. S. KLEINER. St. Louis, Mo., The C. V. Mosby Co., 1954, 746 pp., $7.50

This well-known text has been revised in many aspects in keeping with the recent rapid advances in biochemistry.

The discussions of blood coagulation, enzymes and coenzymes, biologic oxidations, cholesterol metabolism, urea formation, and action of insulin have been completely rewritten. There also has been extensive revision of other sections and the recent knowledge gained through the use of isotopes is found almost throughout.

The author has kept his original purpose of creating a useful, understandable, and compact volume for use as a text in the standard courses in biochemistry. The subject matter is presented clearly. For the more inquisitive student, each chapter contains a well-rounded reference list of pertinent papers.

Laboratory Instructions in Biochemistry (ed. 4)

I. S. KLEINER and L. B. DOTTI. St. Louis, Mo., The C. V. Mosby Co., 1954, 285 pp., $3.50

This excellent laboratory manual is the laboratory partner of Kleiner's *Human Biochemistry*. The directions are set forth clearly and concisely and the experiments for illustrating the points in the subject matter will give the student clear-cut results, which in turn emphasize the reaction under study.

The fourth edition has been revised to include experiments with ion exchange resins, paper chromatography, the phosphatase test for pasteurization, and the Somogyi-Nelson true blood sugar procedure.

The practicing clinical chemist will find this manual useful as a ready reference for rapid toxicologic determinations and for procedures not "routine." The Appendix contains a rather complete list of formulas for the preparation of the reagents and solutions. It is the reviewer's opinion that the formulas could have been made part of the specific procedures in order to acquaint the student with the contents of the reagent used, the time required in its preparation, and any necessary precautions, even though in course work these reagents are provided on the shelf.

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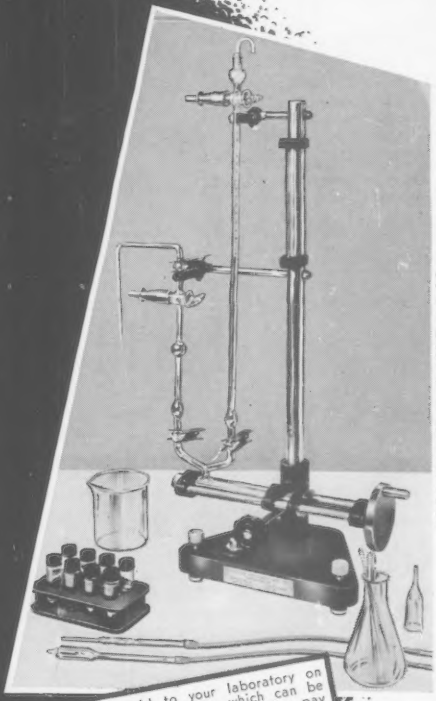

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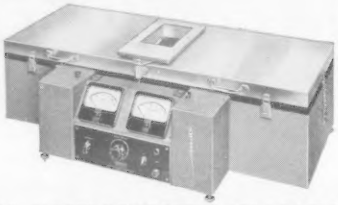

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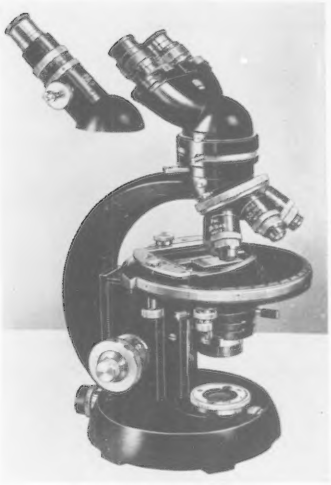

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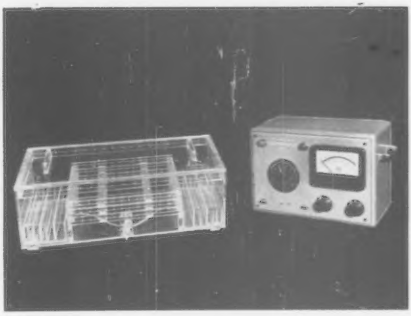

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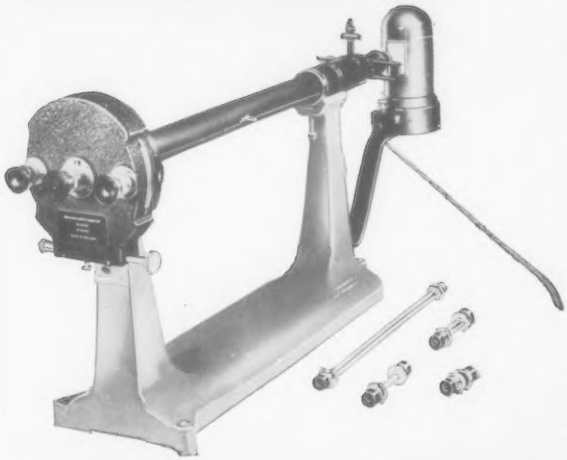

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